University of Alberta

Effects of feeding increasing proportions of barley grain on metabolic, immunologic, and productive responses of early lactating dairy cows

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Animal Science

Department of Agricultural, Food and Nutritional Sciences

Edmonton, Alberta
Spring, 2007

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Your file Votre référence
ISBN: 978-0-494-29948-7
Our file Notre référence
ISBN: 978-0-494-29948-7

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DEDICATION

This thesis is dedicated to my parents and my brothers for giving me the support and love all the way through. To me this means a lot and I can't express in words how grateful I am to you all. I hope I made you proud.

ABSTRACT

The present thesis studied two situations related to enhanced release of endotoxin in the rumen fluid and its translocation through gastrointestinal tract. In the first experiment, eight Holstein cows received four different amounts of barley grain at 0, 15, 30, and 45% (on dry matter basis) in a 4 x 4 Latin square design. Feeding increasing amounts of barley grain proportionately increased the concentration of endotoxin in the rumen fluid, increased plasma concentrations of several acute phase proteins, and altered the plasma concentration of several metabolites and minerals. In the second experiment, rumen and colon samples from feedlot cattle were tested for changes in the permeability to ^{3}H-mannitol with or without presence of lipopolysaccharide (LPS). Permeability of rumen and colon mucosa to ^{3}H-mannitol increased 4-5 folds at lower pH values and in presence of LPS. Translocation of LPS across the rumen and colon mucosa was independent of pH.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
APP	Acute phase proteins
APR	Acute phase response
ARA	Acute ruminal acidosis
BHBA	Beta hydroxyl butyric acid
CD	Cluster differentiation
CRP	Complement reactive protein
DM	Dry matter
DIM	Days in milk
DMI	Dry matter intake
ELISA	Enzyme linked immunosorbent assay
HDL	High density lipoproteins
Hp	Haptoglobin
HSL	Hormone sensitive lipase
IL	Interleukin
LAL	Limulus amoebocyte lysate
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MT	Metallothionine
NEB	Negative energy balance
NEFA	Non esterified fatty acids
PTH	Parathyroid hormone
SAA	Serum amyloid A
SARA	Sub acute ruminal acidosis
SEM	Standard error of mean
TAG	Triacyl glycerides
TNF	Tumour necrosis factor

Chapter 1

General introduction

The transition period, consisting of 3 wk before and 3 wk after parturition, is a critical period for the health and productivity of a dairy cow. During this period, two major events occur: parturition and initiation of lactation. The beginning of lactation imposes considerable stress on the dairy cow and most of the metabolic disorders such as acidosis, milk fever, ketosis, retained placenta, laminitis, fatty liver, displaced abomasum, and infectious diseases like metritis and mastitis do occur during this period of time. Although many hypotheses have been proposed during the years to explain the causes of metabolic disorders, their etiology and pathogenesis still remain unclear.

Most of the metabolic disorders encountered immediately after parturition are related to human imposed changes in the feeding practices and management of the dairy cow. Dairy cows as ruminants have evolved with the ability to digest roughage; however, to support high milk production they require high intake of dry matter very rich in energy. Since forage-based diets are not rich in energy, diets containing high amounts of grain are being fed to cows. On the other hand, the ruminant digestive system is not developed to digest high amounts of starch; therefore feeding high proportions of grain causes a variety of alterations in the rumen environment. Three major changes have been shown to occur in the rumen during feeding of high-grain diets as follows: 1) a rapid decline in ruminal pH, 2) major changes in microbial ecology, and 3) an increase in the concentration of endotoxin, a component of the outer cell wall of all Gram-negative bacteria. The decline

in rumen pH is associated with acute or subacute rumen acidosis also known as ARA and SARA, respectively. Changes in microbial populations include increased numbers of lactic acid producing bacteria and decreased numbers of lactate-utilizers. Also, the number of starch-utilizers, mostly Gram-negative bacteria, increases. This is associated with the release of great amounts of endotoxin in the rumen environment. Studies indicate that endotoxin increases up to 20-fold in cattle fed high-grain diet compared to cattle receiving a forage-based diet (Nagaraja et al., 1978; Andersen et al., 1994). Presence of endotoxin in the rumen fluid is associated with enhanced toxicity. For example, the injection of ruminal fluid from cattle on high-grain diets to mice or calves is associated with death of the experimental animals (Nagaraja et al., 1978; 1979). Evidence indicates that endotoxin translocates into the bloodstream and causes a variety of metabolic, immunologic, and clinical responses (Andersen et al., 1994). Although several epidemiological studies have shown a strong correlation between the amount of concentrate fed and the occurrence of multiple metabolic disorders like acidosis, fatty liver, ketosis, left-displaced abomasum, and laminitis in dairy cattle (Cappock, 1972; Dougherty, 1976; Nagaraja, 1978; Ametaj et al., 2005) the reasons underlying these associations are not clear. A growing body of evidence suggests a role for endotoxin in the etiology and pathogenesis of multiple metabolic disorders.

The implication of endotoxin in the etiology of metabolic disorders comes from several lines of evidence. For example, induction of ruminal acidosis by overfeeding high-grain diets showed clinical and biochemical changes similar to those observed in experimental endotoxemia and the signs were linked to translocation of endotoxin from the gastro-

intestinal tract (Aiumlamai et al., 1992). Fatty liver which is thought to be related to negative energy balance was induced by over-feeding highly fermentable carbohydrates and it was shown to be highly correlated to activation of the inflammatory response (Ametaj et al., 2005). Gram-negative infections causing mastitis, metritis, and laminitis are also associated with fatty liver in dairy cattle (Higgins and Anderson 1983; Jorritsma et al., 2000; Zerbe et al., 2000). Similarly, acute cases of milk fever show clinical signs similar to those of endotoxemia and studies support translocation of endotoxin from gastrointestinal tract during milk fever and parturition (Aiumlamai et al., 1992). The aforementioned studies implicate endotoxin as the common factor in multiple metabolic disorders. Most of the metabolic disorders in dairy cattle are related to alteration of carbohydrate, lipid, and mineral metabolism and recently it has been found that the mediators of the inflammatory response are also related to metabolic disorders (Ametaj et al., 2002; 2005).

In the present study, we feed dairy cows different proportions of barley grain and attempt to evidence whether there are inflammatory, metabolic, mineral, ingestive, and productive alterations associated with feeding of increasing proportions of barley grain to early lactating dairy cows. We also measure changes in the ruminal fluid that might be related to feeding of different proportions of barley grain. In an *in vitro* experiment we attempt to understand whether acidotic conditions, similar to that of rumen and gastrointestinal acidosis, affect the permeability of rumen and colon tissues to endotoxin. Results indicate that feeding increasing proportions of barley grain to early lactating dairy cows is asscociated with major alterations in rumen fluid, mineral, carbohydrate, and

lipid metabolism as well as with induction of an inflammatory response. Also, incubation of rumen and colon tissues in Ussing chamber under acidotic conditions altered their permeability to a large non-nutrient molecule such as mannitol. Presence of endotoxin in the solution also affected the permeability of rumen and colon to endotoxin.

Chapter 2

Literature Review

2.1. Consequences of feeding high-grain diets to ruminant animals

Ruminant animals are primarily herbivores with little or no starch in their diet. Ruminants themselves do not produce fiber-degrading enzymes, but they harbor bacteria, fungi, and protozoa that digest fiber. The host provides the microorganisms with a suitable habitat for growth, and the microbes supply protein, vitamins, and short-chain organic acids for the animal. Microbial protein accounts for as much as 90% of the amino acids reaching the small intestine, and energy from short-chain organic acids (primarily acetic, propionic, and butyric acids) drives animal metabolism (Nocek and Rusell, 1988).

Ruminal microorganisms can also ferment starch and sugars, and these nonfibrous materials increase fermentation rate and animal productivity (Nocek and Rusell, 1988). However, when ruminants are fed fiber-deficient rations, homeostatic mechanisms of digesta flow, gas removal, and pH regulation are disrupted, and the animal can be severely affected (Allen, 1997; Rowe, 1999). Overfeeding cattle with diets rich in starch results in several disorders such as SARA, ARA, fatty liver, laminitis, liver abscesses, displaced abomasum, and bloat (Dunlop 1972; Nocek 1997, Russell and Rychilik, 2001). The mechanism(s) by which grain increases susceptibility of ruminant animals to metabolic disorders is not clear.

Sub-acute ruminal acidosis is a prevalent disorder in most dairy herds. The effects of SARA on individual cows include decreased DMI, decreased efficiency of milk production, low milk fat, unexplained diarrhea, poor body condition, impaired cow health, and high rates of involuntary culling (Nocek, 1997; Kleen et al., 2003). It is estimated that SARA costs the North American dairy industry between $500 million and $1 billion (U.S.) annually, with the costs per affected cow estimated at $1.12 (U.S.) per day (Mutsvangwa, 2003). Economic losses caused by SARA result from decreased efficiency of milk production, increased veterinary bills, and premature culling. Since profitable milk production requires highly fermentable carbohydrates, in order to replace energy lost through milk and to counteract the negative energy balance (NEB) in early lactation, managing the disease rather than eliminating it has been suggested in high producing dairy herds (Nocek, 1997). Due to this reason, in contrast to ARA, SARA is generally regarded as a herd rather than an individual problem. Feeding high-grain diets is associated with low rumen fluid pH and a major shift in ruminal ecology.

2.2. Ruminal microbial changes during feeding of high-grain diets

Ruminant animals and ruminal microorganisms have evolved together for millions of years, and the rumen is inhabited by diverse and interdependent populations of bacteria, protozoa, and fungi. Because ruminal microorganisms are highly competitive, the ruminal community is normally quite stable. However, humans have drastically altered the diet that ruminants consume. When cattle are fed high-grain diets the rumen becomes severely acidic (ruminal pH < 5.5), and ARA is caused by the overgrowth of starch-fermenting, lactate-producing bacteria (*Streptococcus (S.) bovis* and *Lactobacillus* ssp.) (Owens et al.,

1998). If the dietary shift is gradual, *Megasphera (M.) elsdenii* and *Selenomonia* (S.) *ruminantium* convert lactic acid to acetate and propionate, the ruminal pH is not as severely affected (Owens et al., 1998), and the ruminal ecology is not so drastically altered (Tajima et al., 2000). However, even high concentrations of volatile fatty acids (VFA) can cause SARA (Tajima et al., 2000), and pH-sensitive ruminal bacteria (e.g., cellulolytics) are inhibited if the ruminal pH is < 6.0 (Russell and Wilson, 1996).

A variety of microbial changes have been documented over the years associated with feeding of high-grain diets. For example, there is a marked increase in the number of *S. bovis* (Gram-positive bacterium) which utilizes the carbohydrates to produce lactic acid and lowers the rumen pH. The resulting low pH destroys the fibre-digesting cellulolytic bacteria and protozoa (Russell and Rychlik, 2001). Low rumen pH also enables the lactic acid bacteria to utilize carbohydrates and produce lactic acid which further lowers rumen fluid pH (Slyter, 1976).

During high-starch diets, there is a shift in bacterial population from predominantly fiber-digesting cellulolytic bacteria to starch-digesting amylolytic bacteria. Mackie et al. (1978) reported an increased amylolytic bacterial population in grain adapted animals. In experimentally induced SARA, in hay-adapted and grain-adapted cattle, the increase in population of amylolytic bacteria in the rumen was more consistent in grain-adapted group compared to hay-adapted one (Goad et al., 1998). Furthermore, lactate-utilizing bacteria increase in the rumen as cattle are adapted to high-grain diets. *M. elsdenii*, a Gram-negative bacterium, is found to be increased in cattle that are adapted to high-grain

diets. Butyrate production by *M. elsdenii* serves as an electron sink during the oxidation of lactate to pyruvate (Counott et al., 1981). Furthermore, another lactate-utilizing bacteria, *Butyriovibrio fibrosolvens* (Gram-negative bacterium), has been shown to predominate during adaptation to high-grain diets (Mackie and Gilchrist, 1979). All Gram-negative bacteria release a considerable amount of endotoxin in their surroundings as part of their matrix and against their competitors. Endotoxin, an outer cell wall component of all Gram-negatives, is a very harmful toxin against all mammals including ruminants.

2.3. Sources of endotoxin in dairy cattle

Endotoxin, otherwise known as lipopolysaccharide (LPS), is a cellular component of Gram-negative bacteria and is an extremely potent toxin. The main toxic moiety of the LPS is the lipid A, which is responsible for the pathophysiological changes associated with endotoxin and Gram-negative infections. Endotoxin is released when bacteria grow or when bacterial cell wall is damaged. Endotoxin is encountered on feedstuffs and other sources of animal feed (Lindgren et al., 1988). Endotoxin is also released in the infected uterus and hoofs, as well as in the mammary gland, from Gram-negative bacterial infections and translocates into the bloodstream (Smith et al., 1985; Dohmen et al., 2000; Stokka et al., 2001). Several studies have shown that when high concentrate diets are fed to cattle the concentration of endotoxin in the rumen fluid increases dramatically. For example, Nagaraja et al. (1978) reported a 20-fold increase in the concentration of endotoxin in the rumen fluid, whereas Gozho et al. (2006) reported a 300% increase when high-grain diets were fed to cattle compared to forage-fed cattle. Because rumen

epithelium lacks mucous secretions, that normally protect mucous membranes, exposure to acidic environment causes inflammation, ulceration, and scarring of rumen walls (Owens et al., 1998). Chronic acidic pH also causes the epithelium to release oxidants such as metalloproteinases that cause tissue damage as well as thickening and inflammation of rumen wall known as rumenitis, hyperkeratosis, and keratosis (Rowe, 1999).

2.4. Translocation of endotoxin into the blood circulation

There is no clear evidence of how endotoxin is translocated into the blood. However, there are several hypotheses explaining the possible method of translocation of endotoxin into the blood, and the presence of endotoxin in the blood has been demonstrated previously. It has been suggested that the acidic rumen environment, changes in osmotic pressure, and ruminal endotoxin resulting from feeding easily fermentable carbohydrates may render the rumen epithelium susceptible to injury and translocation of rumen endotoxin into the prehepatic bloodstream (Brent, 1976; Enemark et al., 2002; Kleen et al., 2003). Similarly, feeding high-grain diets to calves results in microlesions of ruminal papillae (Kay et al., 1969; McManus et al., 1977), which may result in translocation of endotoxin into the bloodstream. Presence of endotoxin in the blood was demonstrated in cattle and sheep after induced grain engorgement (Dougherty et al., 1975; Andersen et al., 1994). Similarly it was demonstrated that endotoxin can be absorbed from the gastro-intestinal tract of normal animals and animals under shock as well as from ligated segments of swine intestine containing endotoxin in the lumen (Truszczynski and Pilaszek, 1969; Gans and Matsumoto, 1974; Olofsson et al., 1985). The response of host

to endotoxin is extremely complex and involves interaction between endotoxin and blood components that may increase or inhibit the actions of endotoxin.

2.5. Immune responses associated with feeding of high-grain diets

Several investigations indicate that feeding high-grain diets to dairy or beef cattle is associated with the release of a variety of acute phase proteins (APP) (Ametaj et al., 2005, Gozzho et al., 2005; 2006). For example, Jafari et al. (2006) reported that feeding backgrounding and finishing feedlot cattle with high proportions of grain is associated with increased plasma concentrations of serum amyloid A (SAA), lipopolysaccharide-binding protein (LBP), and haptoglobin. As well, abnormal high amounts of alpa$_1$-acid glycoprotein (α_1-AGP) were found in both backgrounding and finishing cattle (Jafari et al., 2006). These proteins are released by the liver as the first line of defense against translocation of bacterial toxins or bacteria.

When there is disturbance of homeostasis in conditions like inflammation, infection, and tissue damage the organism initiates a nonspecific defense mechanism before the specific defense mechanisms come into play. This early and nonspecific protective mechanism is called the acute phase response (APR). The APR is known to contribute to the host defense by neutralization of infectious agents, minimization of tissue damage, and participation in tissue repair, and regeneration. Macrophages and monocytes are the most common blood cells associated with initiating the cascade of events during the APR. Bacterial products such as endotoxin and other events like mast-cell degranulation and aggregation, or induced platelet activation can activate macrophages and monocytes

directly resulting in the production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6. In addition, fibroblasts and endothelial cells also produce proinflammatory cytokines. These proinflammatory cytokines are involved in the initiation of APR.

Broadly, the APR involves the febrile phase and alterations in the biosynthetic profile of the liver. The febrile phase is a protective mechanism and is mediated by the cytokines through the induction of PGE_2 In the second phase, there is rapid alteration in the biosynthetic profile of the liver. Under normal conditions, the liver synthesizes plasma proteins and maintains them at a steady state in blood. Liver also supplies the necessary components for immediate defense at the site of injury, confining tissue destruction, clearing harmful agents, and aiding in tissue repair. Under emergency conditions, the cytokines TNF-α IL-1, and IL-6 stimulate the synthesis and secretion of a group of plasma proteins by the liver through interaction with the hepatic cell surface receptors. This group of plasma proteins constitutes the acute phase proteins (APP) and has protective effects like inactivation of proteases, support the process of wound healing, and scavenging of free oxygen radicals. The APP can be divided into two classes: (1) class 1 [α_1–AGP, C-reactive protein (CRP), haptoglobin, and serum amyloid A (SAA)] which are increased during an APR and class 2 APP (α_2–macroglobulin and cx_1-antichimotrypsin) that are suppressed during the APR. Class 1 APP is induced by IL-1 or a combination of IL-1 and IL-6, whereas class 2 APP is mainly regulated by IL-6 and glucocorticoids. During APR, most of the class 1 APP are induced between 50% and several fold over the normal concentrations whereas major APP like SAA can increase

over 1000-fold above normal concentration. The majority of APP is produced by hepatocytes with some being produced by the endothelial cells, fibroblasts, enterocytes, and adipocytes. The four common APP studied in cattle are SAA, LBP, haptoglobin, and CRP.

Serum amyloid A. Proteins of the SAA family are apolipoproteins synthesized primarily by the liver. They are found predominantly associated with high-density lipoprotein (HDL) in plasma, with different isoforms being differentially expressed. The proinflammatory cytokines IL-1, IL-6, and TNF-α induce the secretion of SAA from the liver (Jensen and Whitehead, 1998). During conditions of inflammation, the concentrations of SAA increase by more than 1000-fold within 24 h of initiation of inflammation (Hoffman et al., 1982; Marhaug, 1983). Although synthesized primarily in the liver, extrahepatic expression of SAA also has been documented. The SAA has a beneficial role in host defenses and plays an important role during the APR to an inflammatory stimulus such as infection, tissue injury, and trauma. Furthermore, SAA associates with the fraction of high density lipoprotein (HDL)-3 and replaces apolipoprotein A-1 (apoA-1) (Eriksen et al., 1980). Although the precise mechanism of SAA action in host defense is not properly understood, several functions have been proposed including detoxification of endotoxin, inhibition of lymphocyte and endothelial cell proliferation, inhibition of platelet aggregation, and inhibition of T lymphocyte adhesion to extracellular matrix proteins (Urieli-Shoval et al., 2000). In addition, SAA has been reported to bind to neutrophils and inhibit the oxidative burst response, suggesting that it may help prevent oxidative tissue damage during inflammation (Linke

et al., 1991; Gatt et al., 1998). As well, SAA has been shown to suppress immune responses to antigens *in vitro* by affecting T cell–macrophage interactions and helper T lymphocyte function (Aldo-Benson et al., 1982; Benson and Aldo-Benson 1979). In addition, SAA is a potent anti-inflammatory molecule and it has been shown to inhibit fever induced by IL-1 and TNF-α in mice (Shainkin-Kestenbaum et al., 1991). The proinflammatory cytokines IL-1, IL-6, and TNF-α also stimulate the secretion of SAA in the intestinal epithelial cells and it has been proposed that these are involved in the local defense mechanisms against endotoxin in the gut.

Intravenous administration of bacterial endotoxin results in a steep increase in the concentration of SAA in plasma and the systemic response is dose dependant (Jacobsen et al., 2004). Increased plasma SAA is also observed during exposure to endotoxin from infection of the mammary gland (Lehtolainen et al., 2004). In these conditions, there is both a systemic increase and an increase in the amount of SAA in the milk. Similarly, subcutaneous injection of endotoxin results in synthesis of SAA by enterocytes and cells of the lamina propria. However, the production is more pronounced in cells of lamina propria indicating that SAA is also involved in neutralizing the gut derived endotoxin.

In cattle, rumen is an important source of endotoxin and feeding high-grain diets results in several-fold increase in concentration of endotoxin in rumen fluid (Nagaraja et al., 1978; Anderson et al., 1994). Gozho et al. (2005; 2006) induced SARA in steers and observed an inflammatory response evidenced by increased concentrations of endotoxin in the rumen as well as in plasma.

Lipopolysaccharide-binding protein. The LBP is a protein synthesized primarily by hepatocytes in response to the proinflammatory cytokines IL-1 and/or IL-6 (Tobias et al., 1999; Schumann and Latz, 2000). The principal function of LBP has been suggested to be the transferring of lipids such as endotoxin (i.e., LPS) to macrophages or lipoproteins. The LBP does that by first dissociating the endotoxin aggregates into endotoxin monomers. Then, LBP facilitates the transfer of endotoxin to endotoxin receptors on monocytes and neutrophils also known as membrane-associated cluster differentiation (mCD14) receptor (Schumann et al., 1990; Wright et al., 1990). Furthermore, the LBP helps in neutralization of endotoxin by catalyzing the movement of endotoxin monomers from endotoxin aggregates to HDL (Schumann et al., 1990). During infections by Gram-negative bacteria or sepsis, where there is excess endotoxin in the circulation, LBP binds to the lipid A part of the endotoxin and transfers it to the mCD14 present on monocytes and macrophages. This results in the phagocytosis and clearance of endotoxin from circulation (Lengacher et al., 1996; Grunwald et al., 1996). However, excess endotoxin signaling can lead to an exaggerated host response eventually resulting in the development of septic shock (Dinarello, 1997). Several studies have proved that the binding of CD14 and LBP to endotoxin accelerates the detoxification of this molecule by preventing its ability to induce an exaggerated proinflammatory response (Flegel et al., 1993; Hubsch et al., 1993; Levine et al., 1993). More recently, several lines of evidence indicate an enhanced detoxification and removal of endotoxin through LBP-mediated transfer of LPS to chylomicrons (Harris et al., 1990, 1991; Vreugdenhil et al., 2003).

There is considerable evidence suggesting a protective role of LBP in mediating the host response to endotoxin to combat infections by Gram-negative bacteria. When LBP knock out mice were infected with the Gram-negative bacterium *Salmonella typhimurium,* they showed an increased susceptibility compared to their wild-type counterparts (Jack et al., 1997; Fierer et al., 2002). Similarly, providing an exogenous source of LBP protected mice from septic shock elicited by endotoxin or *E. coli* infection (Lamping et al., 1998). Also, survivors of septic shock exhibit a higher level of plasma LBP compared to non-survivors indicating a critical role of LBP in combating the highly proinflammatory molecule endotoxin (Opal et al., 1999).

In cattle, 40% of the naturally occurring mastitis is caused by Gram-negative bacteria and the mammary gland which is highly sensitive to endotoxin, develop mastitis when exposed to endotoxin (Erskine et al., 1991). Endotoxin is detectable in the milk of cows with *E. coli* mastitis with significant absorption of endotoxin into the systemic circulation (Anri, 1989; Katholm and Andersen, 1992). Increased amounts of LBP are produced by cattle in response to intramammary endotoxin (Bannerman et al., 2003). Studies correlating increased concentrations of LBP to gut derived endotoxin are lacking and although there are no direct data relating LBP to endotoxin from grain feeding, Emmanuel et al. (2007) reported an acute phase response in cattle experiencing SARA due to highly fermentable carbohydrates and which had direct-fed microbials in the diet.

Haptoglobin. Haptoglobin is another APP produced by the liver. Haptoglobin has been shown to bind free hemoglobin which in the free form is toxic, proinflammatory, and

causes kidney damage (Wagener et al., 2001). By binding to hemoglobin, during conditions of hemolysis, haptoglobin prevents bacteria from using the iron to grow (Wassell, 2000). The haptoglobin molecule is a tetramer, consisting of two alpha and two beta chains. The alpha and beta chains arise from proteolytic processing of the same precursor. Each beta chain can bind an alpha-beta heterodimer of hemoglobin so that each haptoglobin tetramer binds one hemoglobin tetramer and then the hemoglobin-haptoglobin complexes are removed by the reticuloendothelial system. In addition to uptake of hemoglobin, haptoglobin has antioxidant and anti-inflammatory properties (Langlois et al., 1996; Arredouani and Ceuppens, 2002). Furthermore, haptoglobin has immunomodulatory effects such as inhibition of granulocyte chemotaxis, phagocytosis, bactericidal activity, and mast cell proliferation (Rossbacher et al., 1999; El-Ghmati et al., 2002). These effects are mediated through binding of haptoglobin to the CD11/CD18 receptor of the immune cells. As well, haptoglobin also has been shown to suppress T-lymphocyte responses and B-cell mitogenesis in response to endotoxin (Baseler et al., 1983). Serum concentrations of haptoglobin increase in cattle in clinical conditions like mastitis and respiratory diseases and has been used as a diagnostic marker for these conditions (Heegaard et al., 2000; Eckersall et al., 2001).

Haptoglobin is normally undetectable in healthy cattle and concentrations become detectable only when bacteria are present in the bloodstream leading to an inflammatory reponse (Deignan et al., 2000). Administration of glutamine around parturition reduced plasma concentrations of haptoglobin in postparturient cows compared to control cows (Jafari et al., 2006). Since postparturient cows are susceptible to infection and

translocation of bacteria due to lowered immune function the authors suggested that glutamine reduces translocation of bacteria. These experiments show that haptoglobin is an indicatior of bacterial translocation or presence of bacteria in blood. Plasma concentrations of haptoglobin also increase when cattle experience SARA and Gozho et al. (2005) observed a significant increase in the concentration of haptoglobin when animals experienced SARA and the intensity of the haptoglobin response increased as the time below pH 5.6 increased. The amount of grain in the diet has a significant effect on plasma concentration of haptoglobin and cattle fed increasing amounts of grain in the diet responded by eliciting a more intense APR evident by increased concentrations of haptoglobin (Gozho et al., 2006; Jafari et al., 2006).

C-reactive protein. The CRP is a highly conserved APP produced in the liver and belongs to the pentraxin family of proteins (Du Clos et al 2000). C-reactive protein is very sensitive to inflammation and its concentration in plasma increases to varying degrees in response to inflammation, trauma, or infection. C-reactive protein plays important roles in protection against infection, clearance of damaged tissue, prevention of autoimmunization, and regulation of the inflammatory response (Mold et al., 2002). Five identical subunits arranged as a cyclic pentamer constitutes the structure of CRP. The binding face (B face) of CRP has receptors for ligands and interacts with two calcium atoms. Phosphocholine which is found on a wide variety of pathogenic bacteria and on phosphatidylcholine in cell membranes is the prototypic ligand for CRP (Volanakis and Kaplan, 1971). Opsonization of foreign particles, activation of complement by binding to C1q, and binding to FcγR on phagocytic cells to mediate phagocytosis are the important

functional activities of CRP (Kaplan and Volanakis 1974; Crowell et al., 1991). The site that interacts with C1q and FcγR are present on the opposite or A face of CRP (Agarwal and Volanakis, 1994).

C-reactive protein has an important role in host defense against infection. C-reactive protein has the capacity to bind to bacteria and activate the complement pathway resulting in efficient removal of the pathogen (Volanakis and Kaplan, 1971; Volanakis, 1982). There is direct evidence from model systems that CRP can protect against Gram-negative bacteria like *Haemophilus influenzae* (Lysenko et al., 2000). C-reactive protein also contributes to resistance against the lethal toxicity of Gram-negative bacterial endotoxin. Mice expressing high levels of transgenic rabbit CRP are partially protected against lethal challenges by mediators of septic shock including endotoxin, platelet activation factor (PAF), and the combination of TNF-α plus IL-1β, (Xia and Samols, 1997).

Furthermore, CRP induces the production of pro-inflammatory cytokines such as IL-1 and TNF-α and inhibits chemotaxis and the respiratory burst of neutrophils (Mortensen and Zhong, 2000; Du Clos and Mold, 2001). C-reactive protein is an important APR in humans with plasma concentrations increasing from less than 1 μg/ml up to 500 μg/ml during an inflammatory response. C-reactive protein has been identified in a variety of invertebrate and vertebrate species and there is extensive amino acid homology between the proteins isolated from different vertebrates (Pepys and Baltz, 1983). Although CRP is recognized as an important APP in humans and several other animal species, in cattle

CRP has not been studied extensively. Schrohl et al. (1995) reported the normal CRP concentrations in healthy cattle to be between 16 and 148 ng/mL. Inflammatory conditions, such as mastitis increase CRP concentration by almost 10-fold compared to normal healthy cattle (Schrohl et al., 1995).

2.6. Metabolic changes associated with feeding of high-grain diets

2.6.1. Effects of high grain diets on carbohydrate metabolism

Ruminants are mainly dependent on gluconeogenesis in the liver and kidney as a source of glucose since glucose of dietary origin makes little net contribution to the glucose supply (Bergman et al., 1994; Reynolds et al., 1994). Glucose is the main precursor for synthesis of milk lactose and decreased availability of glucose can limit milk production in cows under negative energy balance (NEB). Mammary gland accounts for 60 to 85% of the total glucose used by lactating ruminants and of the total glucose entering the mammary gland 50 to 85% is used for synthesis of lactose (Elliot, 1976). Availability of glucose and lactose has an impact on milk yield due to the osmoregulatory effect of lactose in the mammary gland. Enhanced lactose synthesis in the mammary gland and its transportation into the lumen leads to enhanced water transport to the lumen and thus milk yield.

Propionate from rumen is the most important substrate for gluconeogenesis (Drackley et al., 2001). Approximately 32 to 73% of the glucose demands are met by propionate absorbed from the rumen (Seal and Reynolds, 1993). Feeding propionate has been shown to increase concentration of plasma glucose and decrease β-hydroxy butyric acid (BHBA)

(Schmidt and Schultz, 1958; Schultz, 1958; Goff et al., 1996). The amount of concentrate in the diet profoundly affects concentration of glucose in blood by supplying more propionate and increased postruminal availability of starch. In cows fed high-grain diets, propionate in the rumen is elevated; however, concentration of acetate and butyrate are lowered (Kennelly et al., 1999). Feeding high proportions of grain to dairy cows has been found to improve their metabolic status. This, also, is associated with increased plasma concentration of insulin and glucose (Jeny and Polan, 1975; Sutton et al., 1988). Similarly, feeding high-grain diets increases the availability of glucose in the intestines and its absorption.

Interestingly, administration of LPS in dairy cows is associated with a biphasic response of plasma glucose; initial hyperglycemia, followed by hypoglycemia several hours later (Werling et al., 1996; Steiger et al., 1999). Release of glucose from liver increases greatly during the hyperglycemic phase of LPS challenge (Naylor and Kronfeld, 1985). Also, LPS-induced secretion of hormones such as adrenocorticotropic hormone (ACTH), cortisol, and activation of the sympathetic nervous system contribute to the LPS-induced changes in carbohydrate metabolism (Boosman et al., 1990; Nonogaki and Iguchi, 1997). In addition, production of cytokines such as IL-1, resulting from interaction between macrophages and LPS, has been shown to increase concentrations of glucagon in plasma which increases plasma glucose by glycogenolysis (Zenser and Powanda, 1977). Furthermore, hypoglycemia associated with translocation of endotoxin results from elevated plasma insulin due to increased glucose utilization and direct insulin-like actions of endotoxin (Filkins and Buchanan, 1977; Wolfe et al., 1977).

2.6.2. Effects of high-grain diets on lipid metabolism

Non-esterified fatty acids (NEFA). The NEFA are stored in adipocytes primarily as triacylglycerols (TAG). Adipocytes are very active metabolically and the stored TAG are hydrolysed and resynthesised constantly. Release of TAG from the adipocytes is initiated by the action of an enzyme known as hormone sensitive lipase (HSL). Other hormones such as epinephrine, norepinephrin, adrenocorticotrophic hormone (ACTH), and glucagon activate, whereas insulin inhibits HSL. Glycerol and NEFA are the two main products of TAG hydrolysis in the adipocytes. Non-esterified fatty acids bind to serum albumin and are transported to target tissues, liver and muscles.

The energy status of dairy cows is highly variable in the periparturient period. Early lactation in dairy cows is often characterized by NEB, as the voluntary DMI is lower than the rate at which nutrients are lost in the milk (Vernon et al., 2002). As a result, cow mobilizes NEFA from adipocytes to support lactation, leading to elevation of plasma NEFA (Grummer, 1995). Plasma concentrations of NEFA are negatively correlated with the energy balance in cows and severe NEB and loss of body weight in cows results in greater serum concentration of NEFA (Rukkwamsuk et al., 1999).

Although NEFA can be utilized as a source of energy by the liver and muscles, during periods of NEB an elevated concentration of NEFA may have pathological consequences. Generally, NEFA in the circulation are taken up by the liver and are either stored as TAG or secreted as very low density lipoproteins (VLDL). However, when high concentration of NEFA persists for prolonged periods of time this leads to storage of TAG in the liver

leading to a pathological condition know as fatty liver. Fatty infiltration of the liver occurs in dairy cows at or around parturition and also precedes ketosis, a disturbance of carbohydrate metabolism (Vazquez-Anon et al., 1994). Several other studies, also, have correlated high concentrations of NEFA in plasma and fatty liver to metabolic disorders like milk fever, retained placenta, and displaced abomasum (Grummer, 1995; Rukkwamsuk et al., 1999).

Interestingly, lipid metabolism, and as a result, plasma NEFA are affected by exposure to LPS. During the initial phase of LPS administration, there is stimulation of lipolysis, as reflected by the increase in concentration of NEFA and glycerol in plasma (Steiger et al., 1999). A variety of other factors, like increases in concentrations of ACTH, cortisol, and catecholamines as well as enhanced activity of the sympathetic nervous system contribute to the lipolytic response to LPS (Boosman et al., 1990; Nonogaki and Iguchi, 1997). In addition, a decrease in the utilization of NEFA by the muscle tissue, contributes to the LPS-induced increase in plasma NEFA (Romanowsky et al., 1980).

Plasma β-hydroxy butyric acid (BHBA). Negative energy balance is prevalent in all cows during early lactation because the energy lost in the milk is accompanied by a decrease in DMI. As a consequence, the cow mobilizes her body resources to support milk production. There is fatty acid mobilization from the adipose tissue in the body and consequently the plasma concentrations of NEFA increase (Grummer et al., 1995). Non-esterified fatty acids incorporated into the liver are converted to TAG and are either burned in mitochondria or released as VLDL. Burning of NEFA generates ketone bodies

such as acetone, acetoacetate, and BHBA. Accumulation of ketone bodies in the blood causes ketosis.

Butyrate is a ketogenic VFA and part of BHBA is also synthesized from absorbed butyrate in the rumen epithelium of ruminants. In cattle 90% of absorbed butyrate carbon is converted to ketone bodies (i.e., mainly BHBA and acetoacetate) prior to release into portal circulation. Rumen butyrate has an effect on plasma BHBA and Andersson and Lundström (1985) found a positive correlation between butyrate intake from silage and milk ketone bodies. Similarly, Krehbiel et al. (1992) infused butyrate into the rumen of steers and reported an increase in blood ketones and a decrease in blood glucose. Ketone bodies formed, may be oxidized in the peripheral tissues or act as lipogenic precursors (Madison et al., 1964). Feeding lactose also increases rumen butyrate and plasma BHBA (Schingoethe, 1976; DeFrain et al., 2004). The amount of starch in the diet has a profound effect on concentration of butyrate in the rumen and increasing amounts of corn starch in the diet causes a linear decrease in the amount of butyrate in the rumen (Krause et al., 2003).

Infection or inflammation is accompanied with marked changes in lipid metabolism and changes in plasma ketone bodies (Nueufeld et al., 1976). Administration of IL-1 and TNF-α alters plasma concentrations of ketone bodies and it has been postulated that the antiketogenic effects of infection and inflammation are mediated through these cytokines (Memom et al., 1992). Endotoxin has an antiketogenic effect in cattle and Waldron et al. (2003) reported a decline in plasma BHBA following LPS administration in dairy cattle.

These authors speculated that the decrease in BHBA was primarily due to inhibition of hepatic ketogenesiss (Huhtanen et al., 1993). The LPS-induced inhibition of ketogenesis in cattle appears to be mediated by cytokines TNF-α and IL-1 as well as insulin (Memon et al., 1992).

Plasma cholesterol. Cholesterol is a major component of cell membranes and constitutes up to 50% of lipids in membrane rafts (Pike, 2004). Cholesterol is important for the maintenance of membrane structure and function. Distribution of cholesterol in the body is carried out by lipoproteins such as low density lipoproteins (LDL) and high density lipoproteins (HDL). The LDL distributes cholesterol to peripheral tissues, mediated by LDL receptors. Even though LDL are the predominant plasma lipoproteins in humans, they account for less than 10% of total lipoproteins in cattle (Bauchart, 1993). Apolipoprotein A-1 is the major lipoprotein in HDL and the primary acceptor for nonesterified cholesterol from the peripheral tissues (von Eckardstein et al., 1993, Forte et al., 1993). High density lipoproteins return excess cholesterol from tissues to the liver, by a process called reverse cholesterol transport (Funke 1997). Cholesterol esters and free cholesterol contribute to the lipid composition of LDL and HDL (Tall et al., 1981; Laplaud et al., 1991).

In addition to their role in transport of cholesterol and lipids, plasma lipoproteins provide an important role in controlling responses of the host to endotoxin (Feingold et al., 1997). Low density lipoproteins and HDL play important roles in neutralizing and removing endotoxin from circulation (Feingold et al., 1997; Sewnath et al., 2000). The LBP

transfers endotoxin to lipoproteins and the lipid A acyl moiety of endotoxin is inserted between lipoprotein phospholipids (Parker et al., 1995; Wurfel et al., 1997). The resulting endotoxin–lipoprotein complex is very stable and results in endotoxin neutralization by preventing endotoxin to bind its receptors and initiate the release of cytokines (Ulevitch et al., 1979; Harris et al., 1991). Hepatocytes remove the lipoprotein-endotoxin complex from the circulation and excrete it into the bile (Munford et al., 1981; Read et al., 1993).

In humans, hypocholesterolemia has been consistent with infection or critical illness and several reports have shown strong correlation between low plasma cholesterol and mortality in critically ill patients (Windler et al., 1994; Fraunberger et al., 1999). Based on these findings, some investigators suggest that low proportions of cholesterol in lipoproteins impair host's ability to bind and neutralize endotoxin, so that more endotoxin is available to induce harmful inflammation (Fraunberger et al., 1999; Gordon et al., 2001). Hypocholesterolemia has also been directly associated with the inflammatory response and proinflammatory cytokines such as IL-6 have been found to lower concentrations of cholesterol by decreasing lipoprotein production and incorporation of cholesterol into lipoprotein particles as well as increasing catabolism of lipoproteins (Ettinger et al., 1995). Cows also develop hypercholesterolemia during early lactation which is accompanied by an increase in LDL, HDL and serum lipids (Ametaj et al., 2005).

2.6.3. Effects of high grain diets on plasma lactate. Lactate is an important intermediate of carbohydrate fermentation in the rumen of cattle fed grain. Different

biochemical pathways convert lactate to acetate, butyrate, or propionate (Wood, 1961). Rumen bacterial flora and pH play a significant role in influencing the relative amounts of individual fatty acids produced (Counotte, 1981). Lactate is present in two isomers in the rumen, the L- and D-form. Contrary to the eukaryotic cells which produce predominantly L-lactic acid, prokaryotic cells, such as bacteria, are able to produce both isomers of lactic acid in substantial amounts. Exposure of cattle to rapidly digestible carbohydrates, without proper adaptation, leads to an accumulation of lactic acid in the rumen leading to increased concentrations of lactate in plasma and onset of metabolic acidosis. Under normal physiological conditions, lactic acid in the rumen is absorbed through rumen wall and contributes to glucose metabolism (Waldo and Schultz, 1956). The rate of absorption of both isomers through rumen wall seems to be equal; however, there are reports that there is several fold increase in the absorption of D-lactic acid than L-lactic acid during acidosis (Dunlop and Hammond, 1965; Huber, 1975; Harmon et al., 1985). During ruminal acidosis, only the D-isomer tends to accumulate in blood and for long, it has been known that in adult cattle the D-isomer of lactic acid is implicated in the metabolic perturbations that occur during ARA (Dunlop and Hammond, 1965; Dunlop, 1972). The proposed mechanism of this accumulation is that the mammalian organism lacks D-lactate dehydrogenase (LDH), while L-lactate is metabolized efficiently by L-LDH (Cammack, 1969). D-lactate crosses the blood-brain barrier and accumulates in the brain, where it causes central nervous system toxicity (Dahlquist et al., 1984; Geishauser and Thunker, 1997).

Infusion of starch into the large intestine of cattle leads to production of lactic acid and significant decline in blood pH. Clinical symptoms of intoxication, characteristic of acute ruminal acidosis, have been reported indicating that fermentation of lactic acid in the large intestine contributes considerably to the pathogenesis of lactic acidosis (Zust et al., 2000). Concentration of lactate in plasma is influenced by infections and milk lactate was elevated in cows with mastitis (Davis et al., 2004). Bacterial fermentation in the gut produces D-lactate which normally is not produced or metabolized in mammalian tissues. Increased gut permeability leads to translocation of bacterial products in blood and it has been demonstrated in rodents that increased gut permeability is associated with high concentrations of plasma D-lactate (Murray et al., 1994).

Also, elevated concentrations of plasma lactate are indicative of sepsis or endotoxemia in different species (Giri et al., 1990; Olszanecki and Chlopicki, 1999). Administration of endotoxin to rats or pregnant cows is accompanied with increased concentrations of lactate (Giri et al., 1990; Olszanecki and Chlopicki, 1999). The lactic acidemia observed during endotoxemia is related to increased anaerobic metabolism of glucose in skeletal muscle (Shackleford et al., 1986; James et al., 1996)

2.7. Effects of high grain diets on mineral metabolism

Much attention is being paid by dairy nutritionists to the energy and protein requirements of transition dairy cows which has a direct impact on milk production. However, the role of minerals in the transition period and on subsequent production has not drawn considerable attention. Minerals are inorganic elements required by the body for optimum

growth, productivity, and proper muscle and nerve function. In addition, they are essential components of body enzymes, hormones and cells. Minerals, required by dairy cows for optimum health and productivity, can be grouped as macrominerals and microminerals. Macrominerals are needed in greater amounts and are usually expressed as a percentage of the ration DM. Calcium, phosphorus, magnesium, and potassium are macrominerals that are important to the dairy cow. Microminerals are needed in smaller amounts and are generally expressed as parts per million (ppm). Iodine, zinc, iron, copper, molybdenum, fluorine, cobalt, selenium, and chromium are microminerals needed by the dairy cow.

Mineral requirements by dairy cows depend on a number of factors like body size and physiological status like pregnancy, lactation, and growth. The impact of mineral deficiency is not immediate and may take a considerable amount of time for the effects to be noticed. Generally, the problems will be first noticed in the areas of animal health and nutrition. Absorption of minerals is lower than most other nutrients and presents another problem in meeting the mineral requirements of dairy cattle. There are also numerous interactions between minerals which influence the absorption of a particular mineral. Known interactions between minerals include copper-molybdenum, sulfur-selenium, calcium-phosphorus, calcium-zinc, calcium-manganese, iron-manganese, and potassium-magnesium.

Effect of mineral supplementation to dairy cattle has had variable responses in terms of reproduction and performance (Underwood and Suttle, 1999). While Olson et al. (1999)

reported no difference in reproductive performance in cows supplemented with organic or inorganic form of minerals, Stanton et al., (2000) reported that cows receiving organic forms of trace minerals had higher pregnancy rates than those receiving inorganic forms. The improved reproductive performance in organic mineral supplemented dairy cows were attributed to the improved repair of damaged uterine tissue following calving and increased bioavailability of minerals for metabolism (Wedekind et al., 1992; Paripatananont and Lovell, 1994).

Plasma calcium (Ca^{2+}). Calcium is the most abundant mineral in the body and is required for normal functioning of a wide variety of tissues and physiologic processes. Approximately 98% of Ca^{2+} functions as a structural component of bones and teeth. The remaining 2% is distributed in extracellular fluids and soft tissues, and is involved in such vital functions as blood clotting, muscle contraction, nerve transmission, and as second messenger regulating the actions of many hormones (Horst, 1986). Calcium homeostasis results from an intricate balance of input, output, and Ca^{2+} recycling and vertebrates have evolved a highly complex endocrine system that maintains plasma and extracellular Ca^{2+} concentrations in a narrow range.

The onset of lactation places a sudden large demand on Ca^{2+} homeostatic mechanisms of the dairy cow. A cow producing 10 kg of colostrum will lose an amount of Ca^{2+} which is equivalent to 9 times as Ca^{2+} as that is present in the entire Ca^{2+} pool of the cow. Calcium lost from the plasma pool is usually replaced by increasing intestinal Ca^{2+} absorption and bone Ca^{2+} resorption. Blood Ca^{2+} does not truly reflect Ca^{2+} status of the cow because it

is maintained in a narrow range of 9-11 mg/dL by homeostatic mechanisms. The concentration of ionized Ca^{2+} in plasma is regulated largely by parathyroid hormone (PTH). Lower concentrations of plasma Ca^{2+} stimulate secretion of PTH which acts on the kidney and on bone to increase the concentration of plasma Ca^{2+} and stimulates production of 1, 25-dihydroxy vitamin D_3. Elevated concentrations of plasma Ca^{2+}, in turn, depress secretion of PTH. Calcium absorption from the lumen of intestines is by passive diffusion between the intestinal epithelial cells and by active transport across the epithelial cells which require 1, 25-dihydroxy vitamin D_3. The 1, 25-dihydroxy vitamin D_3 increases Ca^{2+} absorption from the intestine and, in conjunction with PTH, increases Ca^{2+} resorption from bone. (Bronner, 1987; McDowell, 1992). Elevated concentration of plasma Ca^{2+} stimulate production of the hormone calcitonin which inhibits the production of PTH.

Calcium requirements of dairy cows are influenced by such factors as age, weight, type, and stage of production and the amount of Ca^{2+} absorbed is affected by chemical form, source of Ca^{2+}, interrelationships with other nutrients, and animal requirements. Forages are generally good sources of Ca^{2+} and legumes are higher in Ca^{2+} content than grasses. The absorption of alfalfa Ca^{2+} is much lower than absorption of Ca^{2+} from corn silage when fed to dairy cows. Cereal grains are, in general, low in Ca^{2+} and when high-grain diets are fed to dairy cattle, they require supplementary sources of Ca^{2+}. In cattle fed high-concentrate diets, dietary Ca^{2+} in excess of requirements, improves gain or feed efficiency (Bock et al., 1991).

Milk fever or parturient paresis results in dairy cows when endocrine system fails to provide enough Ca^{2+} to prevent development of hypocalcaemia. Hypocalcaemia increases the susceptibility of dairy cows to infectious disease and it has been documented that cows with milk fever are more likely to contract mastitis (Curtis et al., 1985). The importance of Ca^{2+} in immune function is also well known. Calcium is required for a wide range of functions in immune cells including adhesion, motility, gene expression, proliferation, and activation of calcineurin (Berridge, 1993; Ghosh and Greenberg, 1995). Calcineurin activates different genes that control immune response and cell growth. Several studies have investigated effects of hypocalcemia on immune function. Interestingly, Kehrli and Goff (1989) reported that hypocalcemia did not exacerbate immune functions in hypocalcemic cows. However, activation of the immune system has a negative impact on the macromineral concentrations in blood. Immune activation following administration of LPS decreases concentration of Ca^{2+} in plasma and this finding suggests that immune activation may have important implications on Ca^{2+} homeostasis and metabolic health of dairy cows (Waldron et al., 2003).

Concentration of Ca^{2+} in blood is affected by exposure to LPS. Thus, administration of LPS to midlactating dairy cows decreases Ca^{2+} concentration 2h after infusion of LPS (Waldron et al., 2003). In porcine endotoxemic models, hypocalcemia is observed and a marked decrease in concentration of Ca^{2+} in blood is seen within the first 2 h of LPS infusion (Carlstedt et al., 2000). Concentration of Ca^{2+} in plasma is also found to be decreased in naturally occurring Gram-negative coliform mastitis (Wenz et al., 2001). The mechanism that decreases blood calcium during exposure to LPS is not well

understood. Although administration of LPS has been shown to inhibit the function of 1, 25-dihydroxy vitamin D_3 and lower secretion or action of PTH in patients with sepsis (Pramanik et al., 2004), there is no convincing evidence that these factors are responsible for hypocalcemia observed after LPS challenge.

Plasma iron (Fe^{2+}). Iron is an essential component of hemoglobin and myoglobin, the key proteins involved in oxygen transport and utilization. A number of mammalian enzymes like cytochromes and iron-sulphur proteins contain iron and are involved in the electron transport chain (McDowell, 1992). Approximately, two-thirds of body Fe^{2+} is present in hemoglobin in red blood cells and myoglobin in muscle. Hemoglobin contains 0.34% Fe^{2+} which is responsible for proper functioning of every cell in the body. Plasma Fe^{2+} is bound in the ferric state (Fe^{3+}) to a protein called transferrin. Neonatal calves usually have a good body resource of Fe^{2+}, sufficient to prevent anemia if dry foods are fed within a few weeks after birth or a supplemental iron of 40 to 50 mg Fe/kg is fed exclusively on whole milk diet (Bernier et al., 1984). Requirements of older cattle are not well-defined and are probably lower because of the recycling of Fe^{2+} during turnover of red blood cells. Cereal grains normally contain 30 to 60 mg Fe^{2+}/kg with variability seen in forages ranging from 70 to 500 mg Fe^{2+}/kg (Underwood, 1981). The cause of variation in forage Fe^{2+} is due to soil contamination. Often ruminants, on forage based diets, are exposed to high amounts of Fe^{2+} through water forage and or soil ingestion. High dietary Fe^{2+} has been shown to reduce Cu^{2+} status in animals (Humphries et al., 1983).

A deficiency or an excess of Fe^{2+} compromises the proper functioning of the immune system. Plasma Fe^{2+} rapidly falls early in response to bacterial and viral infections and rebounds with recovery. The reduction in Fe^{2+} is attributed to the protective mechanism of the acute phase response. During infections the acute phase protein haptoglobin rapidly binds to hemoglobin and prevents utilization of Fe^{2+} contained in the hemoglobin molecule by bacteria (Wagner et al., 2001).

Infections with Gram-negative or Gram-positive bacteria are associated with a decline in plasma Fe^{2+} as bacteria require Fe^{2+} for their growth and to exert their pathological effects (Bullen, 1981; Lauffer, 1992). To stall bacterial growth and to maintain an Fe^{2+}-free physiological environment mammals employ iron-binding proteins like ferritin and transferrin to lower concentration of extracellular Fe^{2+} to around 10^{-18} M (Bullen et al., 1978; Litwin and Calderwood, 1993). Other iron-binding proteins like lactoferrin and conalbumin block bacteria like *Pseudomonas aeruginosa* by preventing biofilm formation by these bacteria through Fe^{2+} chelation (Singh et al., 2002). Liver has important physiologic roles in maintaining Fe^{2+} homeosatasis and hepcidin, a disulfide-rich peptide produced by hepatocytes is a central regulator of Fe^{2+} metabolism (Park et al., 2001). The main functions of hepcidin include inhibition of (1) intestinal Fe^{2+} absorption (2) placental Fe^{2+} transport and (3) release of recycled Fe^{2+} from macrophages (Nicolas et al., 2001, 2002). The role of hepcidin was unfolded when it was observed that mice injected with LPS or turpentine, to induce an inflammatory state, showed an upregulation of the gene encoding for Fe^{2+} regulatory peptide hepcidin (Niocolas et al., 2002). Similarly, human hepatocyte cultures express hepcidin after exposure to LPS or

medium from LPS activated macrophages. The proinflammatory cytokine IL-6 induces expression of hepcidin as well as the condition of hypoferremia. Among the many cytokines released from macrophages exposed to LPS, IL-6 is the main mediator of hepcidin upregulation during inflammation (Van Amersfoort et al., 2003).

Plasma zinc (Zn^{2+}). Zinc is one of the most important trace elements in the body and is known to participate in the activation of approximately 300 enzymes. Many enzymes, signaling molecules and transcription factors are critically dependent and regulated by Zn^{2+}. Zinc-dependant enzymes are involved in DNA synthesis, cell division, and protein synthesis. Zinc is required for stabilization of the three-dimensional structure of more than 1000 transcription factors involved in the gene expression of various growth factors and steroid receptors (Shankar and Prasad, 1998).

Absorption of Zn^{2+} occurs primarily in the abomasum and small intestine. Zinc absorption is homeostatically controlled and dietary Zn^{2+} is absorbed based on the specific requirements of growth or lactation (Miller and Cragle, 1965; Miller, 1975). Factors like plant species, stage of cut, and soil Zn^{2+} content affects the availability of Zn^{2+} in forages and in general legumes are comparatively richer in Zn^{2+} than grasses (Minson, 1990). Cereal grains contain between 20 and 30 mg/kg of Zn^{2+} while plant protein sources contain about 50 to 70 mg/kg of Zn^{2+}. Proteins called metallothioneins (MT) are known to be involved in the Zn^{2+} homeostasis. These proteins capture Zn^{2+} and release it when there is transient stress to other proteins and enzymes for their optimal function against oxidative stress (Mocchegiani et al., 1998).

Zinc plays important roles in the immune response of the body. Lymphocyte functions such as mitogenesis, antibody synthesis, activation of T-cells, and natural killer cells as well as cellular immunity are regulated by Zn^{2+} (Antoniou et al., 1981; Moulder and Steward, 1989). Improper cellular immunity resulting from Zn^{2+} deficiency can predispose to bacterial, viral, and fungal infections. Thymic atrophy, anergic T-cells, decreased lymphocyte proliferative response, decreased T-helper cells, and reduced thymic hormone secretion are some of other disorders associated with Zn^{2+} deficiency (Falchuk, 1998, Wellinghausen and Rink, 1998).

Although there is a vast array of research related to Zn^{2+} in humans, there has been little research carried out to examine the relationship between Zn^{2+} and the immune response. Furthermore, studies carried out in cattle and sheep are in conflict with the results obtained from human studies. These studies suggest that marginal Zn^{2+} deficiency does not impair cell-mediated or humoral immune responses (Droke and Spears, 1993; Droke et al., 1993).

Interestingly, hypozincemia is observed in response to endotoxemia or inflammation and this is related to redistribution of plasma Zn^{2+} to hepatocytes (Erskine and Barlett 1993). Metallothionein has been ascribed in the regulation of Zn^{2+} homoeostasis and participation in the APR (Cousins, 1985). Hepatic induction of MT occurs in response to inflammation and it has been suggested that the resulting increased supply of exchangeable Zn^{2+} may facilitate the many enzymic processes necessary for mounting the

APR (Cousins, 1985). Increased concentrations of MT in response to inflammation sequester Zn^{2+} to the liver and donate it to Zn^{2+}-dependent enzymes. The latter are necessary for the production of APP and facilitation of metabolic processes in the gluconeogenic/glycolytic pathway when large energy demands are placed on the host during the APR. Interleukin-6, which is proinflammatory and secreted in response to LPS, induces the production of MT in the liver resulting in the sequestration of Zn^{2+} into the liver hepatocytes (Brady et al., 1987). The effects of LPS on circulating levels of Zn^{2+} were studied in normal and MT-knocked out mice. While normal mice exhibit gross hypozincemia, MT-knockout mice have normal concentrations of plasma Zn^{2+} indicating that MT is the principal factor responsible for the decline in plasma Zn^{2+} during APR (Rofe et al., 1996).

Plasma Copper (Cu^{2+}). Copper functions as an essential component of a number of enzymes including lysyl oxidase, cytochrome oxidase, superoxide dismutase, ceruloplasmin, and tyrosinase (McDowell, 1992). Because Cu^{2+} is important for cell proliferation in the bone marrow, the earliest manifestation of Cu^{2+} deficiency are anemia and leucopenia. Copper availability in most cattle feedstuffs is between 1% and 15% (Hemken et al. 1993). The availability of Cu^{2+} in forages depends on the species involved and available Cu^{2+} in the soil (Minson, 1990), but the availability is highly influenced by the presence of Cu^{2+} antagonists such as molybdenum, sulphur, and iron. Sulfide interacts with Cu^{2+} to form thiomolybdates in the rumen (Suttle, 1991). Free Cu^{2+} then reacts with thiomolybdates in the rumen to form insoluble complexes that are poorly absorbed. Some thiomolybdates are also absorbed into the systemic circulation and affect metabolism of

Cu^{2+} (Gooneratne et al., 1989). Thiomolybdates can result in Cu^{2+} being tightly bound to plasma albumin and not available for biochemical functions and they may directly inhibit certain Cu^{2+}-dependent enzymes. Legumes are usually higher in copper than grasses. Cereal grains generally contain 4 to 8 mg Cu^{2+}/kg, and oilseed meals and leguminous seeds contain 15 to 30 mg Cu^{2+}kg. Copper is also poorly absorbed in the well-developed rumen. Absorbed copper is excreted primarily via the bile with small amounts lost in the urine (Gooneratne et al., 1989). Considerable storage of Cu^{2+} occurs in the liver of a healthy cow.

Failure to meet the requirements for Cu^{2+} leads to a variety of clinical conditions like anemia, reduced growth, diarrhea and poor fertility (Underwood, 1981). Copper deficiency also reduces the phagocytic ability of isolated neutrophils and increases the susceptibility to bacterial infections (Woolliams et al., 1986). Poor availability of Cu^{2+} also leads to an impaired glucose and lipid metabolism and depressed immune system. An excess availability of Cu^{2+} more than the required amount leads to toxicity where liver can accumulate large amounts of Cu^{2+} before signs of toxicity are observed. When Cu^{2+} is released from the liver in large amounts, hemolysis, methemoglobinemia, hemoglobinuria, jaundice, icterus, widespread necrosis, and often death occur.

Copper is an integral part of the APP ceruloplasmin whose blood concentrations are highly sensitive to signals induced by the inflammatory processes (Cousins, 1985). Ceruloplasmin is a ferrooxidase that circulates in the blood and functions as a Cu^{2+} transporter that is able to couple 90-95% of the serum Cu^{2+} (Ryden et al., 1884). There

are reports that this protein also has antioxidant function and prove to be beneficial in several pathological conditions. Liver is the major site for the synthesis of ceruloplasmin and the pro-inflammatory cytokines IL-1, IL-6, and TNF-α increase the hepatic synthesis of ceruloplasmin (Gitlin, 1988; Ramadori et al., 1998). Cells of the monocytic lineage are also capable of synthesizing ceruloplasmin in response to the inflammatory cytokines and bacterial cell wall components, suggesting an important role of ceruloplasmin in host defense and APR (Yang et al., 1986).

The development of inflammatory processes causes significant changes in the metabolism of Cu^{2+} in humans and animals. These changes are generally considered as part of the defense mechanism evoked by the organism to bring the inflammatory reaction under physiologic control (Milanino et al., 1989). Inflammatory processes are associated with a significant and dramatic increase in the total blood concentration of Cu^{2+}. The rise in blood Cu^{2+} concentration is accompanied by a concomitant increase in ceruloplasmin concentration and these factors are highly correlated in animals experiencing inflammatory conditions (Conforti et al., 1982). Increased ceruloplasmin was reported in sheep with pulmonary damage as well as during inflammatory conditions in dogs (Pfeffer and Rogers, 1989; Solter et al., 1991). Furthermore, concentrations of ceruloplasmin in plasma are used as indication of inflammation. Endotoxin, a potent stimulator of the APR, causes an increase in plasma ceruloplasmin. Calves administered with LPS showe an increase in concentrations of plasma ceruloplasmin (Conner et al., 1989).

2.8. Literature cited

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Chapter 3

Effects of Feeding Increasing Proportions of Barley Grain on Metabolic, Immunologic and Production Responses of Early Lactating Dairy Cows

3.1. Introduction

Immediately after parturition dairy cows are fed diets containing high proportions of grain. However feeding high amounts of rapidly fermenting carbohydrates decreases rumen pH (Dunlop 1972), alters rumen microbial population (Mackie et al., 1978), and increases the concentration of endotoxin in the rumen fluid. A 4-20 fold increase in the concentration of endotoxin was associated with feeding high grain diets compared to forage based diets (Nagaraja et al., 1978; Gozho et al., 2005). Changes in osmotic pressure, and ruminal endotoxin resulting from feeding easily fermentable carbohydrates may render the rumen epithelium susceptible to injury resulting in translocation of rumen endotoxin into the bloodstream (Brent, 1976; Enemark et al., 2002; Kleen et al., 2003). Similarly, feeding high-grain diets to calves results in microlesions of ruminal papillae (McManus et al., 1978), which may result in translocation of endotoxin into the bloodstream. The presence of endotoxin in the blood was demonstrated in cattle and sheep after induced grain engorgement (Dougherty et al., 1975; Andersen et al., 1994). Endotoxin from grain feeding has been implicated in the development of multiple metabolic disorders like acidosis, fatty liver, laminitis, and sudden death syndrome (Cappock, 1972; Dougherty, 1976; Nagaraja, 1978; Ametaj et al., 2005).

Endotoxin, otherwise known as lipopolysaccharide (LPS), is a cellular component of Gram-negative bacteria and is an extremely potent toxin. Endotoxin is a strong inducer of APR, which is a nonspecific immune mechanism aimed at restoring disturbed homeostasis. It is initiated in response to infection, physical trauma, or malignancy to prevent ongoing tissue damage, isolate and destroy the infective organism and activate the repair processes necessary to restore the host's normal function. During APR there is alteration in the biosynthetic profile of the liver resulting in production of proteins known as APP. The main stimulators of APP production are the inflammation-associated cytokines IL-6, IL-1, TNF-α, and INF-γ which are produced during inflammatory processes (Gabay and Kushner 1999). The APR is characterized by leukocytosis, fever, alterations in the metabolism of many organs as well as changes in the plasma concentrations of various APP (Gabay and Kushner, 1999).

Two APP, SAA and LBP directly participate in the detoxification and removal of endotoxin during an APR. Plasma concentrations of SAA increase up to 1000-fold during an APR and they are believed to substitute apoA-1 fraction of the HDL by 85% (Eriksen et al., 1980; Marhaug, 1983). The SAA, then, binds to endotoxin monomers and the complex is removed by liver macrophages. Lipopolysaccharide-binding protein at lower concentrations activates the innate responses that deal with endotoxin, whereas at higher concentrations catalyzes the transfer of endotoxin to lipoproteins which results in neutralization of the effect of endotoxin to induce inflammatory responses (Gallay et al., 1994). Haptoglobin binds to free hemoglobin and prevents utilization of iron in the hemoglobin by bacteria which require iron for their growth and multiplication (Wassell,

2000). Although haptoglobin does not deal directly with endotoxin its concentration increases in conditions of bacterial translocation (Deignan et al., 2000). The CRP has several functions like opsonization of bacteria and protective effect against endotoxin by interacting with Fcγ receptors on macrophages (Mold et al., 2002; Marnel et al., 2005).

Administration of LPS alters carbohydrate and lipid metabolism in dairy cows. Plasma glucose shows a biphasic response where there is an initial increase followed by a decrease (Nonogaki and Iguchi, 1997). There is an increase in the concentration of NEFA due to lipolytic effect of LPS as well as a decrease in the concentration of BHBA resulting from its antiketogenic effect (Steiger et al., 1999; Waldron et al., 2003). Similary, inflammatory conditions associated with endotoxemia are associated with a decrease in plasma cholesterol and increase in lactate (Giri et al., 1989; Ettinger et al., 1995).

Immune activation by LPS also has its effects on mineral metabolism. Although the mechanism is not well understood, administration of LPS has been found to decrease blood Ca^{2+} concentration. Acute phase response due to LPS decreases circulating levels of Fe^{2+} and Zn^{2+} mainly due to sequestration of these minerals in macrophages and liver (Erskine and Barlett 1993; Nemeth et al., 2003). Interestingly, inflammatory processes are associated with a significant and dramatic increase in blood concentration of Cu^{2+} due to increased hepatic synthesis of ceruloplasmin, a Cu^{2+} transporter (Ramadori et al., 1998).

The objective of the present study was to investigate the effects of feeding increasing proportions of grain on concentration of endotoxin in the rumen and the subsequent alterations in immune responses, plasma metabolites, plasma minerals, and production responses in early lactating dairy cows.

3.2. Materials and methods

3.2.1. Animals and diets. Eight ruminally cannulated primiparous Holstein cows were used in a 4 × 4 Latin square design with two cows in each square and four periods. The squares represent different treatment groups. The experimental period was a 21-d period, with the first 11-d being adaptation period and the remaining 10-d, the measurement period. At the start of the experiment, the cows were at 60 ± 15 days postpartum. All cows had the same base ration which was supplemented with different amounts of barley grain and barley silage to have four different treatment groups receiving 0%, 15%, 30% or 45% barley grain in their ration. The amount of grain in the diet was stepped up or stepped down during the adaptation period. All experimental procedures were approved by the University of Alberta Animal Policy and Welfare Committee and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993). The cows were housed in tie stalls with free access to water at all times and fed once daily at 0800 and milked twice at 0500 and 1600. Daily ration was fed as total mixed ration (TMR) to meet or exceed the requirements of a 680 kg lactating cow as per NRC (2001) guidelines. Estimated protein and energy contents were similar across the different diets. Ingredients and diet composition of the different treatment groups are presented in Table 3.1.

3.2.2. Sample collection

Blood samples were collected from tail vein on days 1, 3, 5, and 10 of the experimental period at 0800 before the morning feeding. On day 10 of the experimental period, blood samples were collected every two hours starting from 0800 until 2000. Blood samples were collected from the coccygeal veins of cows into 10 mL vacutainer tubes (Becton Dickinson, Franklin Lake, NJ) containing sodium heparin anticoagulant. Blood samples were stored in ice and centrifuged within 20 min at 4^0C for 20 min at 3000×g to separate plasma (Rotanta 460 R, Hettich Zentrifugan, Tuttlingen, Germany). Plasma samples were stored at -20^0C until analysis.

Rumen fluid samples were collected on days 1, 3, 5, 7, and 10 of the experimental period as well as on the last day of the measurement period (d 10) before, and at 2, 4, 6, 8, 10, and 12 h after the morning feeding. Samples were collected through the cannulas using a tube fitted with a strainer and a syringe into a 140 mL plastic container. Rumen fluid pH was determined immediately by a mobile pH meter (Accumet™ AP61, Fischer Scientific, Ottawa, ON, Canada). The samples were then centrifuged at 6000×*g* for 15 min and the supernatant was stored at -20^0C until analysis.

3.2.3. Sample analysis

Rumen fluid LPS

Concentration of cell free LPS in the rumen fluid was determined by the LAL assay (Gozho et al., 2005). For use in the assay 1.5mL of thawed sample (at 37^0C) were centrifuged again at 10,000 × *g* for 30 min. The supernatant was then passed through a

disposable 0.22-μm sterile, pyrogen-free filter (Fischer Scientific., Fair lawn, NJ) and diluted 1000-fold using pyrogen-free LAL reagent water (Associates of CAPE COD., E. Falmouth, MA) in pyrogen free test tubes (Associates of CAPE COD. E. Falmouth, MA). Commercially available kits (Associates of CAPE COD., E. Falmouth, MA) were used for the assay. The method and the quantity of reagents described in the kit were modified to have higher standard ranges of 10 ng/mL to 625 ng/mL. Control standard LPS containing 10 ng of LPS/vial (Associates of CAPE COD., E. Falmouth, MA) was used to prepare the standard solutions. Samples were tested in duplicate and the optical density values were read on micro plate spectrophotometer (Spectramax 190, Molecular Devices Corporation., Sunnyvale, CA) at a wave length of 405 nm. Intra-assay coefficient of variation was below 10% for all the assays in this study.

Plasma acute phase proteins

Serum amyloid A. Concentrations of SAA in plasma were determined by an ELISA kit (Tridelta Development Ltd., Greystones C., Wicklow, Ireland) with monoclonal antibodies specific for SAA coated on the walls of the microtitre strips provided. The monoclonal antibodies and the ELISA method for determination of SAA were originally described by McDonald et al., (1991). Samples were initially diluted 1:500 and samples with optical density values above the range of the standard curve were diluted further and reanalyzed. All samples were tested in duplicate and the optical density values were read on microplate spectrophotometer at 450 nm. The detection limit of the assay was 18.8 ng/mL.

Lipopolysaccharide-binding protein. Concentration of LBP in plasma was determined with commercially available multispecies ELISA kit (Cell Sciences, Inc., Norwood, MA). The antibody coated in the wells cross reacts with bovine LBP. Plasma samples were initially diluted 1:1,000 and samples with optical density values lower than the range of the standard curve were tested with a lower dilution. Samples were tested in duplicate and the optical density at 450 nm was measured on a microplate spectrophotometer. Plasma LBP was calculated by extrapolation from a standard curve of known amounts of human LBP.

Haptoglobin. Concentrations of haptoglobin in the plasma were determined by commercially available ELISA kit (Tridelta Development Ltd., Greystones C., Wicklow, Ireland). According to the manufacturer, the detection limit of the assay was 0.25 ng/mL as defined by the linear range of standard curves. All samples were tested in duplicate and the optical density at 630 nm was measured on a microplate spectrophotometer.

C-reactive protein. Plasma concentration of CRP were measured using commercial human sandwich ELISA kits (Alpco Diagnostics., Salem, NH) which cross react with bovine CRP, according to the manufacturer's directions. The minimum detectable concentration of the assay was 1.9 ng/mL. All samples were tested in duplicate and the optical density values were read on microplate spectrophotometer at 450 nm.

Plasma metabolites

Glucose. Concentration of glucose in plasma was quantified by an enzymatic method by commercially available kits (Diagnostic Chemicals Ltd., Charlottetown, PE). Briefly, the procedure involves phosphorylation and oxidization of glucose in samples resulting in the production of NADH which produces a color proportional to the glucose concentration in the sample. All samples were tested in duplicates and the plasma glucose was then determined by reading on a microplate spectrophotometer at an optical density of 340 nm. According to the manufacture's instructions the lower detection limit of the test was 0.06 mg/dL.

Non-esterified fatty acids. Quantitative determination of plasma NEFA was done by an enzymatic colorimetric method using commercially available kits (Wako Chemicals, Richmond, VA). The principle of the test involves acylation of coenzyme A by fatty acids in the sample in presence of acyl-CoA synthetase and production of hydrogen peroxide in presence of acyl-CoA oxidase. Hydrogen peroxide, in presence of peroxidase, permits the oxidative condensation of 3-methyl-N-ethyl-N-β-hydroxy ethyl-0-aniline (MEHA) with 4-aminoantipyrine to form a purple colored adduct which is proportional to the NEFA in the sample. Samples were tested in duplicates and the optical density was measured at 550 nm on a microplate spectrophotometer. The lower detection limit of the assay was 0.50 mEq/L.

Beta-hydroxy butyric acid. Enzymatic Quantitation of BHBA by β-hydroxybutyrate dehydrogenase was used for quantifying plasma concentration of BHBA using a

commercially available kit (Stanbio laboratory., Boerne, TX). The principle of the test involves conversion of BHBA in the samples to acetoacetate and NADH at pH 8.5 by β-hydroxybutyrate dehydrogenase in the presence of NAD. The NADH produced reacts with INT in the presence of diaphorase to produce a color proportional to the concentration of BHBA in the sample. Plasma BHBA was measured in duplicates by reading on a microplate spectrophotometer at an optical density of 505 nm. The lower detection limit of the assay was 125 mmol/mL.

Cholesterol. Plasma cholesterol was measured using commercially available kits (Diagnostic Chemicals Ltd., Charlottetown., PE). The colorimetric method is based on the principle of hydrolyzing the cholesterol esters to free cholesterol and oxidation of free cholesterol to cholest-4ene-3-one with simultaneous production of hydrogen peroxide. The hydrogen peroxide couples with 4-aminoantyrine and p-hydroxybenzoate, in the presence of peroxidase to yield a chromogen whose intensity is proportional to concentration of cholesterol in the sample. All samples were tested in duplicates and plasma cholesterol was determined by reading the optical density values on a microplate spectrophotometer at 505 nm.

Lactate. Plasma concentration of lactate was determined using commercially available lactate assay kits (Biomedical Research Service Center., Buffalo, NY). The principle of the test involves reduction of tetrazolium salt INT in a NADH-coupled enzymatic reaction to formazan which exhibits a red color whose intensity is proportional to concentration of lactate. The lactate standard provided in the kit was diluted to set a

detection range of 125 to 1,000 μM. All samples were tested in duplicates and the lactate concentration was determined by reading the optical density values on a microplate spectrophotometer at 492 nm.

Plasma minerals

Plasma concentrations of Ca^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+} were measured by atomic absorption spectrometry (Spectr AA800, Varian, Sidney, Australia). Plasma samples were diluted 100 times for estimation of calcium and 10 times for Zn^{2+}, Fe^{2+}, and Cu^{2+}. For estimation of plasma Ca^{2+} acetylene with nitrous oxide burner was used and for plasma Zn^{2+}, Fe^{2+}, and Cu^{2+} acetylene burner was used. The upper detection limit of the instrument was at 5.0 mg/L for Ca^{2+}, Cu^{2+}, and Fe^{2+} and 2.0 mg/L for Zn^{2+}.

3.2.4. Statistical analyses

The MIXED procedure of SAS was used to analyse the blood variables with a repeated measures design using the following model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$$

Where μ is the population mean, α_i is a population parameter corresponding to treatment i, βj is the fixed effect of time j, $(\alpha\beta_{ij})$ is the effect of treatment by time interaction and e_{ijk} is the residual error. The covariance structure of the repeated measurements for each variable was modeled separately according to the lowest values of the fit statistics based on the BIC (Bayesian information criteria) and an appropriate structure fitted. The PDIFF option was used in each of the comparisons. Linear and quadratic trends were determined for treatment effects. Significance was declared at $P< 0.05$.

3.3. Results

3.4.1. Rumen fluid endotoxin

Concentration of endotoxin in the rumen fluid increased proportionately as the amount of grain in the diet increased ($P < 0.01$) (Fig. 3.1). The increase was linear from 0% grain group to 45% group ($P < 0.01$). The group fed 45% grain had the highest concentration (8870 ng/mL) and the control group had the lowest (654 ng/mL). The 15% and the 30% group had 790 and 5021 ng/mL, respectively. Both day and day × treatment were significant in increasing the concentration of endotoxin in the rumen ($P < 0.05$ and $P < 0.01$ respectively).

3.4.2. Plasma acute phase proteins

Serum amyloid A. Feeding increasing proportions of barley grain had an effect on the plasma concentrations of SAA ($P < 0.01$) (Fig. 3.2). The group of cows fed 45% grain had the highest plasma concentration of SAA (32,782 ng/mL,) whereas those of the control group (i.e., fed 0% grain) had the lowest concentration of SAA (9,255 ng/mL). There was an interaction between treatment and day ($P < 0.05$); however, days did not affect plasma concentrations of SAA. Furthermore, the control group and the group of cows fed 15% grain had lower concentrations of SAA compared to the groups of cows fed 30 and the 45% grain ($P < 0.01$). As well, plasma concentrations of SAA in groups of cows fed 30 and 45% grain differed from each other ($P < 0.01$), whereas no differences were obtained between the control and the 15% grain groups.

Lipopolysaccharide-binding protein. Feeding diets with high proportions of barley grain affected plasma concentrations of LBP ($P < 0.01$) (Fig. 3.3). The group of cows fed 45% barley grain had twice as high plasma concentrations of LBP (10,056 ng/mL) when compared to the group of cows fed no barley grain (5,707 ng/mL). There was no effect of day of feeding barley on plasma concentration of LBP; however, an interaction between treatment and day was obtained ($P < 0.01$). Concentrations of LBP in plasma were higher in cows fed 45% barley grain compared to those of controls as well as those fed 15 and 30% barley grain ($P < 0.01$). Plasma concentrations of LBP also significantly differed between the groups fed 15% and 45% barley grain ($P < 0.01$).

Haptoglobin. Plasma haptoglobin did not differ among the treatment groups (Fig. 3.4). As well, no effect of day or treatment × day interaction was obtained with respect to plasma concentration of haptoglobin.

C-reactive protein. Plasma concentration of CRP changed with the feeding of increasing proportions of barley grain in the diet ($P < 0.01$) (Fig. 3.5). The group of cows fed 45% barley grain had higher plasma concentrations of CRP (1.34 ng/mL) compared to those of cows fed no grain (1.03 ng/L) ($P< 0.01$). The day of feeding grain had an effect on plasma concentration of CRP ($P < 0.05$) among the groups, whereas no such an effect was obtained for the interaction between treatment × day. The treatment group fed the highest proportion of barley grain in the diet had higher concentration of CRP compared to other groups ($P < 0.01$). No differences in plasma concentrations of CRP were obtained among the groups fed no grain and the groups of cows fed 15 and 30% grain.

3.4.3. Plasma metabolites

Glucose. Plasma glucose was also affected by of the amount of barley grain in the diet ($P < 0.01$) (Fig 3.6). The group of cows fed no barley grain had the lowest concentration of glucose in plasma compared to groups fed 15, 30, and 45% barley grain (60.6 mg/dL vs 64.7, 64.3, and 65.5 mg/dL, respectively) ($P< 0.01$). The day of the experiment also did not affect concentration of glucose in plasma. An interaction between treatment and day was obtained among the different groups ($P < 0.05$). The control group had the lowest plasma glucose compared to the groups fed 15, 30, or 45% grain ($P < 0.01$).

Feeding different proportions of barley grain also affected concentration of glucose in plasma during different time points after the morning feeding in different treatment groups ($P < 0.01$) (Fig. 3.7). Plasma glucose increased as the percentage of grain in the diet increased. However, time elapsed after morning feeding did not have an effect on plasma glucose concentration. The group of cows fed 45% grain had the highest plasma numerical concentration of glucose followed by the groups fed 30 and 15% and the group fed no grain throughout the day. Furthermore, no treatment × hour interaction was obtained with respect to plasma glucose.

Non-esterified fatty acids. Feeding increasing proportions of grain decreased plasma concentration of NEFA in this experiment ($P < 0.01$) (Fig. 3.8). Plasma NEFA was highest in cows fed no grain (335 mEq/L) followed by those fed 15% (212 mEq/L), 30% (182 mEq/L), and 45% grain (138 mEq/L). The day of the experiment had an effect on plasma NEFA ($P < 0.05$) as well as on the interaction between the treatment and day ($P <$

0.05). The control group had the highest plasma concentration of NEFA when compared to other groups ($P < 0.01$). However, there was no difference between the groups fed 15 and 45% barley grain.

Data obtained from intensive sampling during the last day of the experimental period indicated an effect of time after morning feeding on plasma NEFA (Fig. 3.9). Thus, feeding different amounts of grain had an effect on concentration of NEFA during different hours after feeding ($P < 0.01$). Plasma NEFA were highest before the morning feeding and declined during the day in all treatment groups reaching the lowest point at 10 h after the morning feeding. Time after feeding also had an effect on plasma concentration of NEFA as well as on the interaction between treatment and time after feeding. ($P < 0.01$).

Beta-hydroxy butyric acid. Feeding increasing proportions of barley grain affected plasma concentration of BHBA during the experimental period ($P < 0.01$) (Fig. 3.10). The control group had the highest plasma concentration of BHBA (690 mmol/mL) followed by the group fed 15% (523 mmol/mL) and then those fed 30% (511 mmol/mL) grain. The group of cows fed the highest amount of barley grain (45%) had the lowest plasma BHBA (449 mmol/mL). The group of cows fed no barley grain had the highest plasma concentration of BHBA compared to the other groups ($P < 0.01$). No differences in plasma concentration of BHBA were obtained for other groups.

Analysis of plasma samples obtained at different hours after the morning feeding on the last day of the experimental period (d 10) indicated that feeding different amounts of barley grain affected concentration of BHBA in plasma ($P < 0.01$) (Fig. 3.11). In general, plasma concentration of BHBA increased after the morning feeding and remained elevated until 10 h after feeding in all treatment groups. In addition, the control group had the highest concentration of BHBA at different time points after feeding followed by the groups of cows fed 15, 30, and 45% barley grain. Also, time after feeding affected plasma concentration of BHBA ($P < 0.01$). No such effect was obtained for the interaction between treatment and time after feeding.

Cholesterol. Feeding different proportions of grain affected concentrations of cholesterol in plasma ($P < 0.01$) (Fig. 3.12). The group of cows fed 15% grain had the highest plasma cholesterol (287 mmol/L) followed by the control group (280 mmol/L), and the groups fed 30% (272 mmol/L) and 45% (265 mmol/L) barley grain. Furthermore, day had an effect on plasma cholesterol ($P < 0.01$) and so did the interaction between the treatment and day ($P < 0.01$). Also, plasma cholesterol differed between the group fed 45% grain and the groups fed no grain and 15% grain ($P < 0.05$) as well as between those fed 15% and 45% grain ($P < 0.01$).

Different proportions of grain, also, affected plasma cholesterol at different time points after morning feeding ($P < 0.01$) (Fig. 3.13). Plasma cholesterol was lowest in the group fed 45% grain (292 mmol/L) and highest in the control group (335 mmol/L). Time after

feeding affected plasma cholesterol as well as the interaction between treatment and the time after feeding ($P < 0.01$).

Lactate. Although no effect was observed on concentration of plasma lactate in blood with respect to feeding different amounts of barley grain, there was a tendency for plasma lactate to increase with the increasing amount of barley grain in the diet (Fig. 3.14). Thus, plasma lactate was the highest (541 μmol/mL) in the group of cows fed the largest amount (i.e., 45%) of barley grain and the lowest (410 μmol/mL) in the group fed no barley grain. Day or treatment × day interaction did not affect plasma lactate; however, cows in the control group had lower plasma lactate than those fed 45% barley grain ($P < 0.01$). No differences were obtained among other treatment groups.

Time elapsed after feeding affected concentration of lactate in plasma ($P < 0.01$) (Fig. 3.15). The group fed 45% grain had the highest concentration of plasma lactate throughout the day, whereas the control group had the lowest concentration of plasma lactate. The two other groups fed 15 and 30% barley grain, had intermediate concentrations of plasma lactate between that of the control group and the group fed 45% barley grain. During the last day of the experimental period, all the treatment groups had different plasma ($P < 0.01$) lactate among each other except for the groups fed 30 and 45% barley grain.

3.4.4. Plasma minerals

Calcium. Concentrations of Ca^{2+} in plasma were affected by feeding different amounts of grain in the diet ($P < 0.01$) (Fig. 3.16). The group of cows fed the largest amount of barley grain had the lowest concentration of Ca^{2+} in plasma compared to the groups fed lower amounts of grain. Plasma Ca^{2+} generally decreased from d 1 to d 5 in all four treatment groups. There were no effects of the experimental day or the interaction of treatment × day among the different groups. Significant differences in plasma Ca^{2+} were observed between the group of cows fed 15% grain and the groups fed 0, 30, and 45% grain ($P < 0.05$).

Different proportions of grain in the diet affected plasma Ca^{2+} during different hours after feeding ($P < 0.01$) (Fig. 3.17). The groups fed 15 and 30% grain had higher plasma Ca^{2+} (11.0 and 11.1 mg/dL, respectively) followed by the group fed 45% (10.8 mg/dL) and the control group (10.5 mg/dL) during different time points after the morning feeding. The time after feeding and the interaction between treatment and time after feeding had a significant effect on plasma Ca^{2+} ($P < 0.01$).

Iron. Concentration of Fe^{2+} in plasma was affected by feeding of different amounts of grain in the diet ($P < 0.01$) (Fig. 3.18). The highest plasma Fe^{2+} was observed in the group of cows fed 15 and 30% grain (187 and 180 μg/dL, respectively) followed by the control group (164 μg/dL). The lowest plasma Fe^{2+} was observed in the group fed 45% grain (146 μg/dL). Experimental day did not affect plasma Fe^{2+} as well as treatment × day interaction. The group of cows fed 45% grain had lower plasma Fe^{2+} compared to

control group and the groups fed 15 and 30% grain ($P < 0.05$). Similarly, the control group had lower plasma Fe^{2+} than the group fed 15% grain ($P < 0.01$). No treatment differences were observed between the control group and those fed 30% grain as well as those fed 15 and 30% grain.

Intensive sampling on d 10 of the experimental period revealed no treatment differences between the different groups (Fig. 3.19). Individual treatment differences were observed between the control group and the group fed 15% grain ($P < 0.05$), between those fed 15 and 30% grain and those fed 30 and 45% grain ($P < 0.05$).

Zinc. Feeding different proportions of grain did not affect plasma Zn^{2+} (Fig. 3.20). Neither different days nor treatment x day interaction had a significant effect on plasma Zn^{2+}. However, treatment affected plasma Zn^{2+} during different hours after feeding ($P < 0.01$) (Fig. 3.20). The group of cows fed the highest amount of grain (45%) had the highest plasma Zn^{2+} (306 μg/dL) and the control group had the lowest (97 μg/dL). The other two groups fed 15 and 30% grain had plasma Zn^{2+} at 195 and 196 μg/dL, respectively. The control group had lower plasma Zn^{2+} than the groups fed 15, 30, and 45% grain ($P < 0.01$). Also, the group fed 45% grain had higher concentration of Zn^{2+} than the groups fed 15 and the 30% grain ($P < 0.01$).

Copper. Treatment did not affect plasma Cu^{2+} in different treatment groups during different days (Fig 3.21). However, a day effect ($P < 0.01$) and an interaction between the treatment and the experimental day were obtained ($P < 0.01$). Feeding increasing

proportions of grain in the diet increased ($P < 0.01$) plasma Cu^{2+} at different time points after the morning feeding (Fig. 3.23). There was a dose-dependant increase in plasma Cu^{2+} from 119 μg/dL in the control group to 142 μg/dL in the group fed 45% grain. The groups fed 15 and 30% grain had 129 and 134 μg/dL, respectively. Interaction between treatment and time after feeding affected plasma Cu^{2+} ($P < 0.01$) while no effect of time after feeding on plasma Cu^{2+} was obtained.

3.4.5. Rumen fluid pH, feed intake, and milk production

Rumen fluid pH. Treatment had an effect in lowering the pH of the ruminal fluid ($P < 0.01$) (Fig 3.24). The lowest overall ruminal fluid pH was observed in cows fed 45% grain diet (pH 6.5) and the highest pH value was obtained for the group fed no grain (pH 6.8). The other two groups fed diets containing 15 and 30% grain had an overall pH value of 6.7. Day had no effect on ruminal fluid pH, and so was the treatment × day interaction. The highest-grain group (45%) had lower ruminal fluid pH than the control and the groups consuming 15 and 30% grain diets ($P < 0.01$).

Data obtained at different time points after the morning feeding, on the last day of the experimental period (d 10), indicated an effect of diet on lowering the ruminal fluid pH during the day (Fig. 3.25). Generally, the ruminal fluid pH declined after the morning feeding and remained low up to 10 h after feeding. Time after feeding had a significant effect on ruminal fluid pH ($P < 0.01$); however, no interaction between treatment and hour was observed. The control group had higher ruminal fluid pH than the treatment groups ($P < 0.01$). No significant differences were observed among the treatment groups.

Feed intake. Increasing the quantity of grain in the diet lowered the total feed intake by the cows ($P < 0.01$). Feed intake was comparable between the control group and the group fed 15% barley grain diet. Both groups of cows fed the highest grain diets (i.e., 30% and 45%) had lower feed intake compared with the group fed no barley grain and the group fed 15% grain ($P < 0.01$). Day as well as treatment × day interaction did not have an effect on total feed intake. Dry matter intake increased with increasing amounts of grain ($P < 0.01$) (Fig. 3.26). Interaction between treatment and day was not significant with respect to DMI.

Milk production. Feeding increasing proportion of barley grain increased milk yield in different treatment groups ($P < 0.01$) (Fig. 3.27). The group of cows fed no grain had the lowest daily milk yield (27.17 kg/day). The total milk yield, in the groups of cows fed 15 and 30% grain was comparable (28.18 and 28.99 kg/day, respectively), whereas the group fed 45% grain had the highest total daily milk yield (31.04 kg/day). Experimental day affected total daily milk yield ($P < 0.05$). No treatment × day interaction was obtained for milk yield. The control group had the lowest milk yield compared to the treatment groups ($P < 0.01$). Among treatment groups, cows fed 45% barley grain had higher milk yield than those fed the 15 and 30% grain diets ($P < 0.01$). Both groups of cows fed the 15 and 30% grain diets differed with respect to milk yield ($P < 0.01$).

3.5. Discussion

3.5.1. Rumen fluid endotoxin

Results of this study are in agreement with our working hypothesis that feeding increasing proportions of barley grain to early lactating dairy cows is associated with increased concentrations of endotoxin in the rumen fluid. Indeed, feeding diets containing 30 and 45% barley grain was associated with 8- and 14-fold increase, respectively, in the amount of endotoxin in the rumen fluid compared to that of cows receiving no barley grain. Results of our study also demonstrated no differences in the amount of endotoxin in the rumen fluid between the groups fed 0% and 15% barley grain.

Our findings also are consistent with previous studies demonstrating enhanced toxicity (i.e, endotoxin content) of rumen fluid from feedlot cattle fed diets containing high-proportions of corn grain (McManus et al., 1978; Nagaraja et al., 1978a, b). In a recent study, Gozho et al. (2005) report no differences in the concentration of endotoxin in the rumen fluid of Jersey steers in which SARA was induced by feeding 3 different diets containing wheat-barley pellets and chopped alfalfa hay in the ratio 2:3, 1:1, and 3:2, (Gozho et al., 2005), although an increase in the concentration of endotoxin from day 1 to day 4 was demonstrated. In a more recent study, Gozho et al. (2006) report a 4-fold increase in the concentration of endotoxin in the rumen fluid when steers were moved from an all-forage diet to a 61% concentrate diet. Interestingly, they failed to detect any changes in the amount of endotoxin in the rumen from all-forage to 41% concentrate diet. In the present study, although the groups of cows fed 0% and 15% barley grain did not differ with respect to concentration of endotoxin in their rumen fluid, a proportional increase was observed when the amount of barley grain was enhanced from 0 to 30% (8-fold) or 45% (14-fold).

Results of our study indicate that endotoxin is present in the rumen fluid even in cows that did not receive barley grain. On the other hand, the reason for increased release of endotoxin during feeding of high-grain diets is not very well understood. Even though Nagaraja et al. (1978) suggests that most of endotoxin in the rumen may come from rapidly growing Gram-negative bacteria or from death of Gram-negative bacteria, they found no changes in the proportion of Gram-negative bacteria in the rumen fluid. Similarly, the total coliform counts in the rumen did not differ when cattle were fed either high-forage or high-concentrate diets (Tkalcic et al., 2000; Gozho et al., 2006). Thus, the reason for the presence of endotoxin in the rumen fluid or for the increased concentration of endotoxin during feeding of diets rich in readily-digestable carbohydrates is not clear.

We propose two sources that might contribute to presence of endotoxin in the rumen fluid in cattle. Firstly, it is known that endotoxin is part of the microenvironment where Gram-negative bacteria live (Donlan and Costerton, 2002). Gram-negative bacteria live in colonies embedded in a matrix of extracellular polymeric substances known as biofilm that is rich in endotoxin (Donlan and Costerton, 2002). Therefore, a basic amount of endotoxin is always present in the environment like rumen where Gram-negative organisms live. Secondly, Gram-negative bacteria have been shown to produce and release structures known as membrane vesicles (MV) during their growth. Membrane vesicles are bilayered spheres that naturally bleb from the outer membrane of virtually all Gram-negative bacteria and contain LPS, phospholipids, hydrolytic enzymes, and outer membrane periplasmic proteins (Li et al., 1996). These structures have bactericidal activity and have been shown to inhibit the growth of Gram-positive bacteria

(MacDonald and Beveridge, 2002). It is possible that the constituents of MV lyse neighboring dissimilar cells so as to increase the nutrient load for the donor cell (Li et al., 1996). Therefore, the increase in the concentration of endotoxin in the rumen fluid with increasing amounts of grain in the diet may be due to the Gram-negative bacteria releasing endotoxin to counteract their close competitors for the available nutrients.

3.5.2. Plasma acute phase proteins

Results of this study demonstrated that concentration of SAA, a key APP, increased in plasma with increasing amounts of grain in the diet. Although plasma concentration of SAA between the groups fed forage alone and 15% barley grain did not differ, a 2- and a 4-fold enhancement was observed when the amount of barley grain was increased to 30% and 45%, respectively. Our findings are consistent with previous reports of increased plasma concentrations of SAA in steers where, SARA was induced by feeding wheat-barley pellets (Gozho et al., 2005) and by increasing the concentrate fraction of the diet above 61%. The plasma concentrations of SAA are also in accordance with the rumen fluid endooxin concentration which increased linearly with increasing amounts of grain in the diet. Serum amyloid A is an acute phase protein produced by hepatocytes in response to cytokines like IL-1, IL-6, and TNF-α triggered during infection, inflammation, and tissue injury (Jensen and Whitehead, 1998). Under normal conditions this protein is present in small quantities and can increase up to 1000-fold during an APR (Marhaug, 1983). Although SAA is said to have a variety of functions during inflammatory conditions, they are believed to substitute apoA-1 fraction of the HDL by 85% (Eriksen

and Benditt, 1980). The SAA, then, binds endotoxin monomers and the complex is removed by liver macrophages.

Our study is the first to report an increase in the plasma concentrations of LBP by feeding increasing proportions of barley grain to dairy cows. We observed a 4-fold increase in the concentration of LBP between the 0% and the 45% barley grain group at the beginning of the experimental period. Similar to SAA, we found a tendency for LBP to decrease towards the end of the experimental period in the higher grain groups. The reason for this decrease is unknown although we did not find a similar trend with the rumen fluid endotoxin concentration. Therefore, the decline in the concentration of LBP is not associated with the decrease in the release of endotoxin in the rumen fluid. This might be related to increased mucosal barrier functions in response to translocation of endotoxin as well as involvement of other neutralizing factors such as albumin, transferrin, and LDL in removal of endotoxin as the dairy cow is adapted to high-grain diets (Gabay and Kushner, 1991; Vreugdenhil et al., 2003).

Lipopolysaccharide-binding protein is an APP synthesized by hepatocytes in response to IL-1 and/or IL-6 that binds to endotoxin present in circulation (Tobias et al., 1999). Several studies suggest a protective role for LBP in mediating the host responses to endotoxin. Studies with mice revealed that LBP knock-out mice were more susceptible to Gram-negative infections by *Salmonella typhimurium* (Fierer et al., 2002). Similarly, administration of LBP protected mice from septic shock induced by LPS or *E. coli* infection (Lamping et al., 1998). Lipopolysaccharide-binding protein at low

concentrations activates and amplifies the inflammatory responses to LPS, thus potentially serving as a critical component in the activation of the innate immune response against this component of Gram-negative bacteria (Gallay et al., 1994). This is achieved by the ability of LBP to transfer LPS to CD14, a glycoprotein found on the cells of monocytic origin (Viriyakosal and Kirkland, 1995). The LPS-CD14 complex then activates Toll-like receptor-4 signaling cascade leading to host cell activation and responses to endotoxin (Ulevitch and Tobias, 1994). Although this system of endotoxin detection is key to mobilization of the host responses to endotoxin, an excess endotoxin signaling can exaggerate the host responses and prove to be deleterious. Recently, it has been shown that at higher concentrations of endotoxin, LBP catalyzes the transfer of endotoxin to lipoproteins which results in neutralization of the effect of endotoxin to induce inflammatory responses (Munford et al., 1981; Levine et al., 1993). Moreover, in presence of soluble CD14 this process is greatly increased (Wurfel et al., 1997). Thus, at lower concentrations of endotoxin, LBP activates the innate responses to deal with endotoxin, whereas at higher concentrations it helps expedited neutralization and removal of endotoxin from circulation.

Although we did not find differences in plasma concentration of haptoglobin among the groups of cows fed different amounts of barley grain, concentration of haptoglobin was high in all groups of cows. Previously we reported values of plasma haptoglobin ranging between 500 and 1,200 μg/mL in transition cows (Jafari et al., 2006). Results of the present study showed haptoglobin concentrations between 400 and 700 μg/mL in control cows and cows fed high grain diets, respectively. Elevated plasma haptoglobin values

suggest translocation of bacteria into the bloodstream of all cows independently of grain feeding. Haptoglobin is released in conditions of hemolysis and binds to free hemoglobin to prevent utilization of iron in the hemoglobin by bacteria which require iron for their growth and multiplication (Wassell, 2000). Haptoglobin is normally undetectable in healthy cattle and concentrations become detectable only when bacteria are present in the bloodstream leading to an inflammatory response (Deignan et al., 2000). Although haptoglobin is not directly related to the presence of endotoxin, concentrations may increase in association with bacterial translocation. Endotoxin or proinflammatory cytokines have been shown to be involved in the loss of mucosal barrier function (Salzman, 1995) and the consequent increased permeability or loss of barrier function can lead to translocation of bacteria and bacterial products (Deitch et al., 1991).

Although there are reports of plasma or milk concentration of CRP in transition cows or in cows with mastitis (Morimatsu et al., 1991; Schrodl et al., 1995; Kruger and Neumann, 1999), our study is the first to report an increased concentration of CRP in cows fed increasing proportions of barley grain. We found a proportional increase in the plasma concentration of CRP by feeding increasing amounts of grain. The increased plasma CRP may be due to translocation of endotoxin into the bloodstream. The CRP has anti-inflammatory properties and protects against endotoxin by interacting with Fcγ receptors on macrophages (Mold et al., 2002).

C-reactive protein is a typical APP that increases up to 1000-fold within 24-48 h of an acute phase stimulus like inflammation, infection, or tissue damage (Volamakis, 2001;

Marnell et al., 2005). C-reactive protein is produced primarily in the liver in response to IL-6. The CRP shares many properties with IgG, including the ability to activate the classical complement pathway (Kaplan and Volanankis, 1974), the capacity to interact with FcγR (Bharadwaj et al., 1999; Marnell et al., 1995) and the ability to bind to ligands. The interaction of CRP with FcγR mediates several functions that are analogous to those of IgG, including cytokine secretion and opsonization of bacteria (Marnel et al., 2005). The ability of CRP to suppress acute inflammation has been well documented in transgenic rodents. For example, mice expressing high concentrations of CRP were protected from lethal shock induced by endotoxin or TNF-α and IL1-β (Xia and Samols, 1997). Also, Mold et al. (2002) has shown that CRP-mediated protection against endotoxin shock requires FcγR associated with induction of IL-10 and suppression of IL-12. Interleukin-10 is a potent inhibitor of TNF-α, IL-1, and IL-12 production and is protective from endotoxic shock.

3.5.3. Plasma metabolites

Results of this study demonstrated that concentration of glucose in plasma was comparable in the groups of cows fed diets containing 15%, 30%, and 45% barley grain in the diet and lower in cows receiving no barley grain during the experimental period. However, diurnal response to feeding increasing proportions of barley grain showed a linear increase in the concentration of glucose as the percent of barley grain in the diet increased. Several reasons might explain the hyperglycemic effect of diets with higher proportions of barley grain. First, high-grain diets are rich in non-structural carbohydrates like sugars, starches, and pectins that are rapidly degraded in the rumen into glucose,

acetate, propionate, and butyrate (Nocek and Tamminga, 1991). Also, grain based diets increase the availability of glucose in the intestines and its absorption into circulation. Second, propionate from rumen is the most important substrate for gluconeogenesis (Drackley et al., 2001). Feeding propionate has been reported to increase concentration of plasma glucose (Schmidt and Schultz, 1958; Schultz, 1958; Goff et al., 1996). Third, administration or translocation of endotoxin into the bloodstream is associated with enhanced plasma glucose because of the stimulatory effect of endotoxin on cortisol and glucagon (Boosman et al., 1990; Nonogaki and Iguchi, 1997).

We observed a decrease in the concentration of NEFA in plasma as the amount of barley grain in the diet was increased during the experimental period. However, analysis of diurnal variation of plasma NEFA revealed no obvious difference between the group fed no grain and the group fed 45% grain. The lower plasma NEFA concentration in cows fed high grain diets may be due to better energy balance through increased propionate production in the rumen. However, if high grain diets provide better energy balance we would expect the same to happen in the diurnal variation of plasma NEFA. But what we observed was lower plasma NEFA concentration in the groups fed 15% and 30% grain and higher NEFA in the 45% group. It is clearly evident that some other factor contributed to elevated NEFA in the high grain group. Lipid metabolism, and as a result, plasma NEFA have been shown to be affected by exposure to LPS. During the initial phase of exposure there is stimulation of lipolysis and increased concentration of NEFA due to a variety of factors like increase in concentrations of cortisol and catecholamines as well as enhanced activity of the sympathetic nervous system (Boosman et al., 1990;

Nonogaki and Iguchi, 1997; Steiger et al., 1999). The increased plasma NEFA in the high-grain group after feeding may be due to an inflammatory response associated with translocation of endotoxin from the rumen. In the late phase, after LPS exposure there is elevated plasma insulin which increases glucose uptake by cells (Filkins and Buchanan, 1977). Insulin has antilipolytic effects which inhibit mobilization of NEFA from adipose tissue. In the present experiment, cows were fed once at 1000 while blood samples were taken at 0800 before morning feeding. It is possible that the antilipolytic effect observed in the late inflammatory response to endotoxin might have partly contributed to the decreased plasma NEFA in blood samples collected before the morning feeding in the high-grain groups.

In the present study cows which did not receive barley grain had the highest plasma concentration of BHBA and decreased linearly as the amount of grain in the diet increased. Also, plasma samples obtained on the last day of experimental period showed an increase in the concentration of BHBA immediately after feeding. There are two sources of butyrate in ruminants. First, plasma BHBA comes from burning of NEFA in liver hepatocytes during conditions of NEB (Baird. 1982). The latter is associated with mobilization of NEFA from adipose tissue (Grummer et al., 1995), burning of NEFA into mitochondria, and release of ketone bodies (i.e., BHBA) in circulation. Second, the amount of butyrate in silage is another source of butyrate for ruminant animals. Thus, Andersson and Lundström (1985) found a positive correlation between butyrate intake from silage and ketone bodies in plasma. In our study cows fed 0%, 15%, 30%, and 45% barley grain had 58, 43, 28 and 13% silage, respectively, on a DM basis. The increased

amount of silage might have partly contributed to the increased plasma concentrations of BHBA in the no grain and lower grain groups. Third, an inflammatory response to endotoxin can also have antiketogenic effects. Waldron et al., 2003 reported a decline in plasma BHBA following LPS administration in dairy cattle which was primarily due to inhibition of hepatic ketogenesis (Huhtanen et al., 1993). The LPS-induced inhibition of ketogenesis in cattle appears to be mediated by cytokines TNF-α and IL-1 as well as insulin (Neufeld et al., 1976; Memon et al 1992). We demonstrated in this study that feeding increasing amounts of grain increased concentration of endotoxin and translocation of endotoxin might have affected plasma BHBA.

Plasma cholesterol concentrations decreased with increasing barley grain in the diet; however, the group fed 15% grain had the highest plasma cholesterol as opposed to the control group. It is not clear why the group fed 15% grain had the highest concentration of choleterol. But, on the last day of the experimental period a linear decrease in the plasma cholesterol was observed as the amount of grain in the diet increased. Cholesterol esters and free cholesterol contribute to the lipid composition of LDL and HDL which are the major carriers of cholesterol to peripheral tissues (Tall et al., 1981; Laplaud et al., 1991). Low density lipoproteins and HDL play important roles in neutralizing and removing endotoxin from circulation (Feingold and 1997). The LBP transfers endotoxin to the lipoproteins resulting in a very stable endotoxin-lipoprotein complex which prevents the release of cytokines (Wurfel et al., 1997; Harris et al., 1990). Hepatocytes remove the lipoprotein-endotoxin complex from the circulation and excrete it into the bile (Munford et al., 1981; Read et al., 1993). In addition to serving as a medium to excrete

neutralized endotoxin from the systemic circulation, bile has an important role in neutralizing gut derived endotoxin and serves as the first line of defense against endotoxin. Lack of bile in the gut lumen is thought to be responsible for an increase in translocation of endotoxin through the intestinal mucosa (Kimmings et al., 2000). Similarly, experimental obstructive jaundice results in endotoxemia, bacterial translocation, and induction of different cytokines (Bemelmans et al., 1992). Since cholesterol is the main precursor of bile we think that the decrease in plasma cholesterol with the high-grain diets may be associated with the need for increased bile secretion. In addition, during inflammatory response, the proinflammatory cytokines IL-1 and IL-6 have been found to lower concentrations of cholesterol by decreasing lipoprotein production and incorporation of cholesterol into lipoprotein particles as well as by increasing catabolism of lipoproteins (Ettinger et al., 1995). We also found an inflammatory response by feeding increasing amounts of grain, which also might have contributed to the decrease of plasma cholesterol.

We showed that feeding increasing amounts of barley grain to dairy cows tended to increase concentration of plasma lactate during the experimental period. However, in blood samples taken at different time points after the morning feeding we demonstrated an increased plasma lactate as the amount of grain in the diet increased. There are two reasons for increased plasma lactate in cows fed high proportions of barley grain: 1) feeding diets rich in readily fermentable carbohydrates is associated with increased concentrations of lactate in the rumen fluid (Counotte et al., 1981) and subsequent absorption of lactate into the bloodstream, and 2) endotoxemia and Gram-negative

bacterial infections are associated with elevated plasma lactate (Davis et al., 2004). For example, administration of LPS to laboratory animals or cattle resulted in increased concentration of plasma lactate (Giri et al., 1990; Olszanecki and Chlopicki, 1999). Moreover, increased concentration of lactate in blood is indicative of sepsis or endotoxemia in a wide variety of species (Giri et al., 1990; Olszanecki and Chlopicki, 1999).

While mammalian cells predominantly produce L-lactic acid, rumen bacteria are capable of producing both the L- and D- isomers (Cammack, 1969). In rodents it has been demonstrated that altered or increased permeability of the gastrointestinal tract mucosa leads to high plasma D-lactate which is of gut bacterial origin (Murray et al., 1994). Therefore, detection of D-lactate in the blood is suggestive of altered gut permeability. It has been proved by several researchers that endotoxin increases the permeability of mucosa by mechanisms including ischemia, hypoxia, and increased production of NO (Rixen et al., 1997; Defazio et al., 1997). Similarly, L-lactic acidosis can also arise from poor tissue perfusion because of endotoxemia and the resulting anerobic glycolysis (Asitz et al., 1988). It is possible that in the present study increased amounts of endotoxin in the plasma associated with high-grain diets may have increased permeability of the gut resulting in increased plasma lactate. In addition, anaerobic glycolysis induced by translocation of endotoxin might have also contributed to the increased plasma lactate.

3.5.4. Plasma minerals

We obtained two different response patterns with regards to plasma calcium concentrations in relation to the amount of barley grain in the diet: 1) a long-term response, and 2) a diurnal response. Thus, during the 10-d experimental period cows fed high proportions of grain in the diets had the lowest plasma Ca^{2+}, whereas, measurements on the last day of the experimental period indicated greater plasma Ca^{2+} in cows receiving the greater amount of grain. The reason for lower plasma Ca^{2+} in cows fed the highest amount of barley grain during the experimental period is not clear; however, translocation of endotoxin into the bloodstream might play a role in lowering plasma Ca^{2+}. Recent studies in dairy cows indicate decreased plasma Ca^{2+} following administration of LPS intravenously (Waldron et al., 2003). The precise mechanism by which LPS lowers plasma Ca^{2+} is not clear; however, earlier studies show that removal of LPS by macrophages is associated with a large increase in Ca^{2+} which is a prerequisite for the release of cytokines and NO (Farber et al., 2005). On the other hand, diurnal increase of plasma Ca^{2+} in cows fed greater amounts of grain might be related to glucose metabolism. Pancreatic β-cell express Ca^{2+} receptors that monitor changes in extracellular Ca^{2+} concentrations and respond by releasing insulin. Although there is not yet a physiological rationale to monitoring of plasma Ca^{2+} by β-cells of the pancreas, activation of these receptors by high plasma Ca^{2+} is associated with stimulation of insulin secretion. Therefore, higher plasma Ca^{2+} in addition to higher plasma glucose in cows fed high-grain diets might be associated with further increase in insulin secretion as a result of binding of Ca^{2+} to pancreatic receptors (Gray et al., 2006).

Plasma Fe^{2+} was found to decrease in the group receiving the highest amounts of grain during the experimental period. Similar to Ca^{2+} data, the group fed no grain diets had lower plasma Fe^{2+} than the groups fed 15% and 30% grain although forages generally contain high Fe^{2+}. No diurnal variation was obtained in the plasma Fe^{2+} between different treatment groups. During infections by Gram-positive and Gram-negative bacteria or endotoxemia the host responds by decreasing plasma Fe^{2+} to stall bacterial growth, as bacteria require Fe^{2+} for their growth and to exert their pathological effects (Bullen, 1981; Lauffer, 1992). Homeostasis of Fe^{2+} is mainly regulated by the hormone hepcidin, a disulfide-rich peptide produced by hepatocytes during inflammatory conditions (Park et al., 2001). In mice, induction of an inflammatory state by injection of LPS showed an upregulation of the gene encoding for hepcidin (Nicolas et al., 2002). Excessive hepcidin production occurs with inflammatory and infectious disorders causing sequestration of iron in the macrophages and inhibition of intestinal iron absorption (Nemeth et al., 2003). The proinflammatory cytokine IL-6 induces expression of hepcidin and among the many cytokines released from macrophages exposed to LPS; IL-6 is the main mediator of hepcidin upregulation during inflammation (Van Amersfoort et al., 2003). Other proteins produced during an acute phase response may also function to decrease Fe^{2+} availability. During an APR haptoglobin rapidly binds to hemoglobin and prevents utilization of Fe^{2+} by bacteria (Waganer et al., 2001). Proteins like ferritin and tranferrin also function to lower plasma Fe^{2+} (Bullen et al., 1978; Litwin and Calderwood, 1993). The decreased plasma Fe^{2+} in cows fed high grain diet may be due to an inflammatory response associated with translocation of endotoxin from rumen.

Plasma Zn^{2+} did not differ by feeding increasing amounts of grain during the experimental period. The group fed 45% grain exhibited a decline from d1 to d 10 which was not the same with the other groups. However, blood samples taken at different time points after feeding tended to have higher plasma Zn^{2+} with increasing amount of grain in the diet. Zinc homeostasis is primarily regulated by MT and conditions like endotoxemia or inflammation decrease concentrations of Zn^{2+} in blood due to sequestration of Zn^{2+} in the liver. The reason for hepatic induction of MT in response to inflammation is that the resulting increased supply of exchangeable Zn^{2+} facilitates the enzymatic processes necessary for mounting an APR (Cousins, 1985). Moreover, in MT-knockout mice administration of LPS did not affect plasma Zn^{2+} while normal mice exhibited hypozincemia in response to LPS indicating the role of MT in lowering plasma Zn^{2+} on LPS exposure (Rofe et al., 1996). Although we expected decreased plasma Zn^{2+} with increasing amount of grain we couldn't see any difference between the groups. Diurnal variation of Zn^{2+} revealed higher plasma Zn^{2+} in the group fed 45% and lowest in the control group. The reason for this response in not clear; however, we speculate that since Zn^{2+} is a constituent of insulin and cows fed high-grain diets had higher plasma glucose, concentrations of insulin may increase in those cows and as a consequence plasma Zn^{2+}.

The development of inflammatory processes causes significant changes in the metabolism of Cu^{2+} in humans and animals. Copper is an integral part of the APP ceruloplasmin whose blood concentrations are highly sensitive to signals induced by the inflammatory processes (Cousins, 1985). Ceruloplasmin functions as Cu^{2+} transporter that is able to couple 90-95% of the serum Cu^{2+} (Ryden et al., 1984; Yang et al., 1986).

Although in the present study we detected an inflammatory response by feeding increasing amounts of grain we were unable to detect significant changes in plasma Cu^{2+} among the different groups during different days. This may be due to the inflammatory responses being waned out by the time when blood sample was collected. Since the feeding was done at 1000 and the samples were collected before feeding the next day, the time difference would have contributed to the failure to observe any changes in plasma Cu^{2+}. However, on the last day of the experimental period when we collected blood samples at different time points after feeding we detected a consistently elevated plasma Cu^{2+} in the high grain groups with the group fed 45% having the highest plasma Cu^{2+}. We also found a linear increase in the plasma Cu^{2+} as the quantity of grain in the diet increased. Inflammatory processes are associated with a significant and dramatic increase in the total blood concentration of Cu^{2+}. The rise in blood Cu^{2+} concentration is accompanied by concomitant increase in ceruloplasmin concentrations and these factors are highly correlated in animals experiencing inflammatory conditions (Feldman et al., 1981; Conforti et al., 1982). Liver is the major site for the synthesis of ceruloplasmin and the pro-inflammatory cytokines IL-1, IL-6, and TNF-α increase hepatic synthesis of ceruloplasmin (Gitlin, 1988; Ramadori et al., 1998). Cells of the monocytic lineage are also capable of synthesizing ceruloplasmin in response to the inflammatory cytokines and bacterial cell wall components, suggesting an important role of ceruloplasmin in host defense and APR. The increase in concentration of Cu^{2+} by increased production of ceruloplasmin is generally considered as part of the defense mechanism evoked by the organism to bring the inflammatory reaction under physiologic control (Milanino et al., 1989). Furthermore, in calves, administration of LPS is associated with increased

concentrations of plasma ceruloplasmin (Conner et al., 1989). The increased levels of plasma Cu^{2+} after feeding may be due to an inflammatory response associated with feeding increasing amounts of grain.

3.5.5. Rumen fluid pH

We were able to see differences in ruminal pH by feeding increasing amounts of grain. Rumen fluid is buffered over a pH range of 5.7 to 7.3 and the rumen pH represents the relative amounts of bases, acids, and buffers. Under neutral conditions, bicarbonate and phosphates are the primary buffers. When the pH falls below 5, VFA and lactate act as buffers (Counotte et al., 1979). The primary end products of microbial digestion in the rumen are the VFA like acetate, propionate, and butyrate as well as lactate. Volatile fatty acids and lactate are absorbed from rumen and serve as a source of energy for the cow. Absorption of VFA and lactate, by removing non-ionized acid and by the exchange of ionized VFA and lactate for bicarbonate during the absorption process aids in maintaining pH near neutrality (Stevens, 1970). Ruminal osmolality normally ranges from 240 to 265 mOsm/L with roughage diets, and 280 to 300 mOsm/L with concentrate diets (Garza et al., 1989). Minerals, VFA, lactate, and glucose are the primary solutes in ruminal fluid contributing to the osmolality. When cattle are fed readily digestable carbohydrates there is more fermentation and production of VFA and lactate and the osmolality of rumen fluid also increases. High osmolality of ruminal contents reduces the rate of acid absorption (Tabaru et al., 1990). This exacerbates acidity and decreases the ruminal pH. Feeding rapidly fermentable carbohydrates also reduces saliva production which also contributes to the increased osmolarity.

In a previous study with increasing amounts of barley grain no differences in rumen fluid pH were observed (Lardy et al., 2004). Similarly, Boyles et al. (1998) also supplemented steers once daily and reported few differences in ruminal pH by feeding increasing levels of barley. The amount of barley, though, was lower in these studies which might be the reason for no obvious difference. However, Reynolds et al. (1993) who fed corn and barley at 30% of the diet noted lower ruminal pH at 0, 4, 16, and 20 h after feeding compared to steers fed only hay. The decreased pH in our study with increasing amount of grain is consistent with previous studies reporting energy supplementation at levels > 30% of the diet decrease ruminal pH (Stewart, 1977; McDonnell et al., 1979; Mould et al., 1983).

3.5.6. Feed intake and milk production

In the present study, feed intake decreased linearly as the amount of grain in the diet was increased. Two potential reasons might explain the feeding response of cows to different amounts of grain in the diet: 1) amount of VFA released in the rumen, and 2) translocation of endotoxin from rumen fluid into blood. Firstly, feeding high-grain diets that are rich in rapidly fermentable carbohydrates to ruminants is associated with increased release of VFA in the rumen fluid and their absorption in blood (Baile and Forbes, 1974). Intraruminal or intravenous infusion of acetate and propionate solutions before or after a scheduled meal is associated with depression of feed intake in cattle (Baile and Forbes, 1974). It is possible that absorption of acetate and propionate in blood and their effect on rumen receptors may have contributed to decreased feed intake in

cows fed high grain diets. The second reason for decreased feed intake with increasing amount of grain in the diet might be related to enhanced release of endotoxin in the rumen, as evidenced in our study, and its translocation into blood. Increased concentrations of endotoxin in the bloodstream and cytokines released after activation of macrophages by endotoxin like IL-6 and TNF-α suppress feed intake in different species (Porter et al., 1998). Waldron et al. (2003) demonstrated reduced feed intake in dairy cows infused intravenously with LPS. The mechanism by which endotoxin causes reduced feed intake in cows is not clear; however, plasma leptin might play a role. Administration of LPS stimulates production of leptin, a hormone that suppresses feed intake in a variety of species (Sachot et al., 2004). In contrast to total feed intake DMI increased with increasing amounts of grain. Forage to concentrate ratio has been shown to affect the DMI intake of dairy cows. Allen (2000) reported a linear increase in DMI with increasing concentrate in the diets. Similarly Llamas-Lamas and Combs (1991) found that DMI was greatest for the diet having the highest amount of concentrate when cows are fed diets with three ratios of forage. Increased DMI with increasing amount of grain in the diet may also be related to high DM in the diet and Lahr et al. (1983) reported a linear increase in the DMI with increasing DM in the feed.

Results of our study showed that milk production was higher in cows fed greater amounts of barley grain. Barley grain contains high nonstructural carbohydrates like sugars, starches, and pectins. Nonstructural carbohydrates are degraded rapidly by rumen bacteria to provide high amounts of glucose and propionate. Glucose is essential for milk synthesis. The availability of glucose to the mammary gland has an important impact on

milk yield because lactose is the major osmoregulator in the mammary uptake of water. Increasing amounts of either post-ruminal glucose or ruminal propionate enhance both milk and protein yield in lactating dairy cows when dietary supply of postruminal starch is low such as with grass silage diets (Rigout et al., 2003). Also, because propionate is the major precursor for hepatic glucose production (Danfaer et al., 1995), increased glucose production and release by the liver may also favor increased milk yield. The increased milk yield may also be due to increased DMI by feeding increasing amounts of barley grain in the present study. Similarly availabilty of glucose and propionate in the rumen and improved metabolic status due to increased plasma concentrations of these nutrients might have contribute to increased milk production associated with high grain feeding.

3.6. Conclusion

In conclusion feeding increasing amounts of barley grain to early lactating dairy cows was associated with an increase in the concentrations of rumen LPS and major APP, alteration of plasma metabolites, minerals, and rumen pH as well as changes in feed intake and milk production.

Table 3.1 Diet composition and ingredients of experimental diets

Ingredients[1] (% of DM)	0% barley	15% barley	30% barley	45% barley
Alfalfa hay	15.00	15.00	15.00	15.00
Alfalfa silage	12.00	12.00	12.00	12.00
Barley silage	58.00	43.00	28.00	13.00
Rolled barley	0.00	15.00	30.00	45.00
Gluten meal	6.00	6.00	6.00	6.00
Fish meal	1.03	1.03	1.03	1.03
Canola meal	0.98	0.98	0.98	0.98
Dairy premix[2]	0.58	0.58	0.58	0.58
Megalac[3]	1.79	1.79	1.79	1.79
Limestone	0.58	0.58	0.58	0.58
Biofos[4]	0.40	0.40	0.40	0.40
Magnesium oxide	0.35	0.35	0.35	0.35
Sodium bicarbonate	0.76	0.76	0.76	0.76
Vitamin E[5]	0.09	0.09	0.09	0.09
Vitamin D_3[6]	0.17	0.17	0.17	0.17
Molasses	0.35	0.35	0.35	0.35
Hydrogenated tallow	1.87	1.87	1.87	1.87
Nutrient composition (% of DM)				
ME (Mcal/kg DM)	2.45	2.42	2.40	2.39
CP	16.20	16.40	16.50	16.70
NDF	32.80	30.20	27.60	25.00
ADF	21.80	19.40	17.00	14.60
NFC	35.40	38.80	42.10	45.50
Ca	1.30	1.30	1.20	1.20
P	0.40	0.40	0.50	0.50
DCAD (mEQ/kg)	300.00	274.00	248.00	223.00

[1]Fed as total mixed ration
[2]Contained Calcium 0.1%, Phosphorous 0.6%, Sodium 11.5%, Magnesium 0.3%, Potassium 0.7%, Sulphur 0.23%, Zinc 5000 mg/kg, Copper 1170 mg/kg, Manganese 3100 mg/kg, Iodine 80 mg/kg, Cobalt 6.2 mg/kg, Vitamin A 1,265,000 IU/kg, Vitamin D 142,000 IU/kg, Vitamin E 3,800 IU/kg
[3]Contained 85% fat as fatty acids and 9.6% calcium with a NE_L of 6.52 Mcal/kg
[4]Contained monocalcium phosphate and dicalcium phosphate in the ratio 2:1
[5]Contained 5,000 IU/kg
[6]Contained 500,000 IU/kg

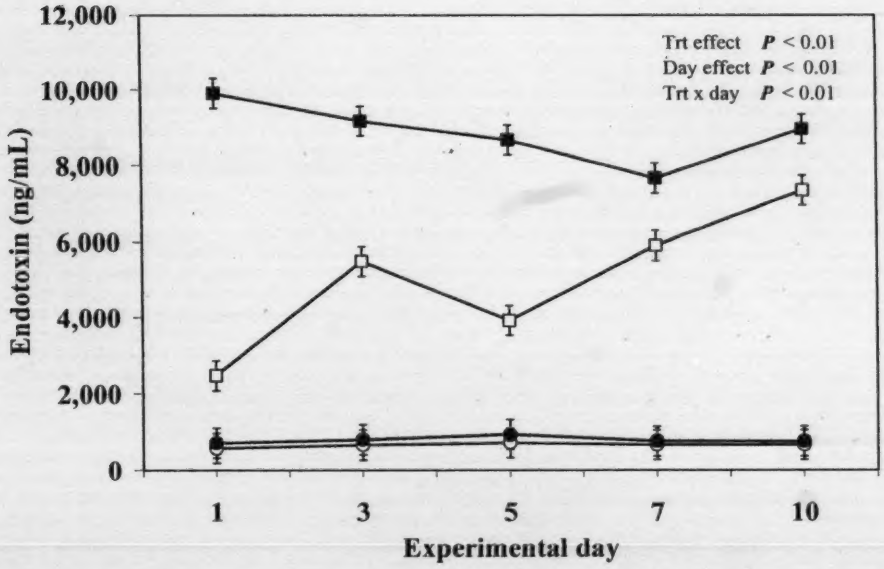

Figure 3.1 Concentration of endotoxin (ng/mL) in the rumen fluid of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

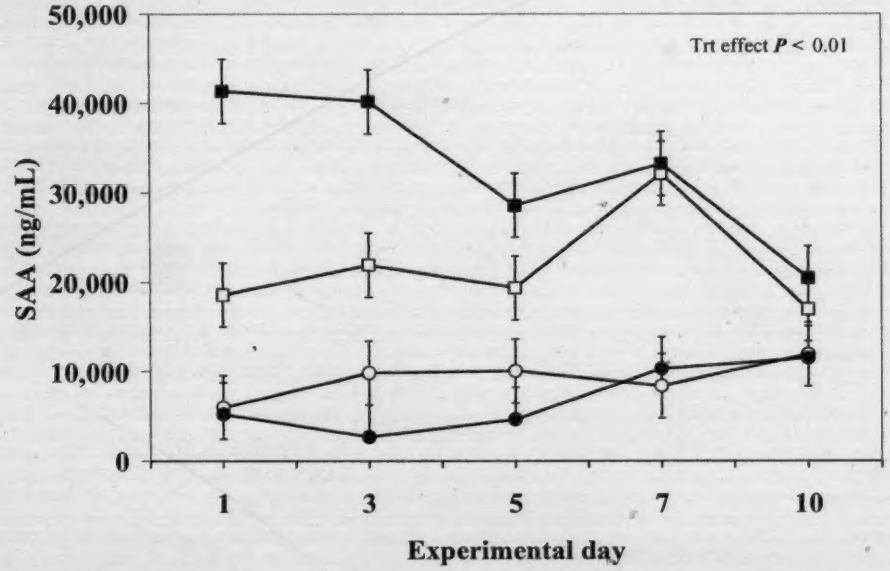

Figure 3.2 Concentration of serum amyloid A (SAA) in the plasma (ng/mL) of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

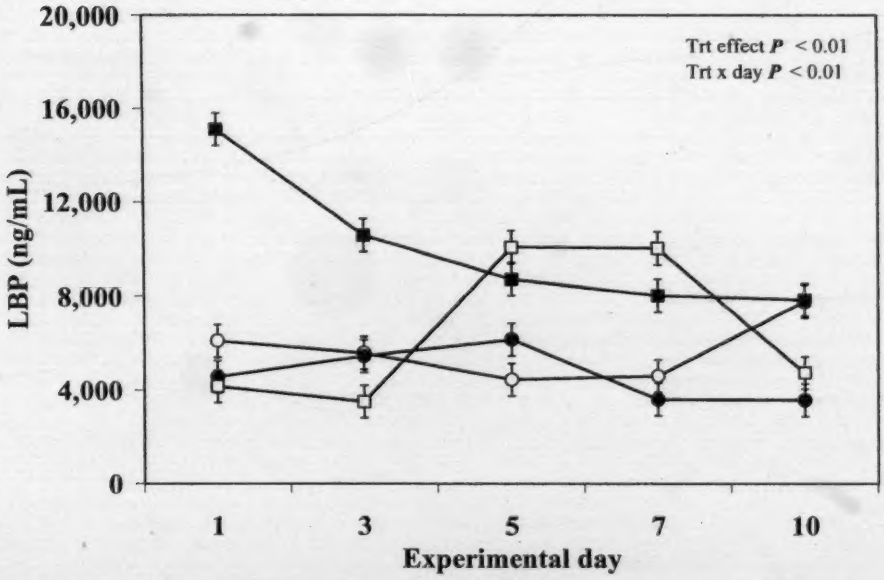

Figure 3.3 Concentration of lipopolysaccharide-binding protein (LBP) in the plasma (ng/mL) of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

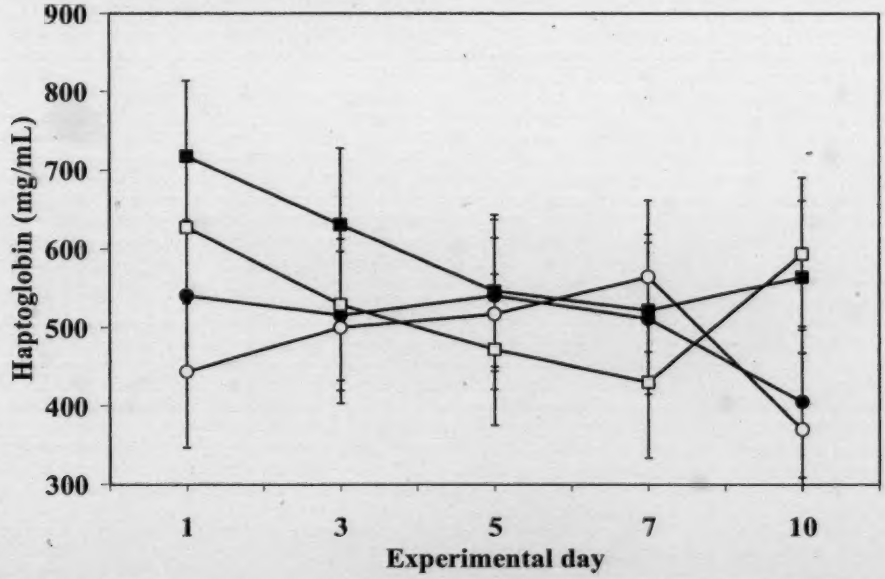

Figure 3.4 Concentration of haptoglobin in the plasma (mg/mL) of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

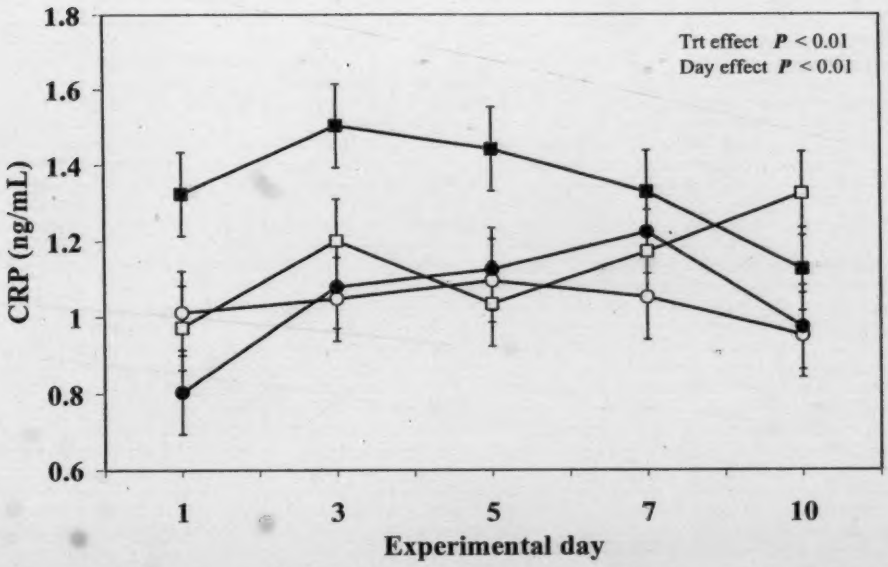

Figure 3.5 Concentration of C-reactive protein (CRP) in the plasma (ng/mL) of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

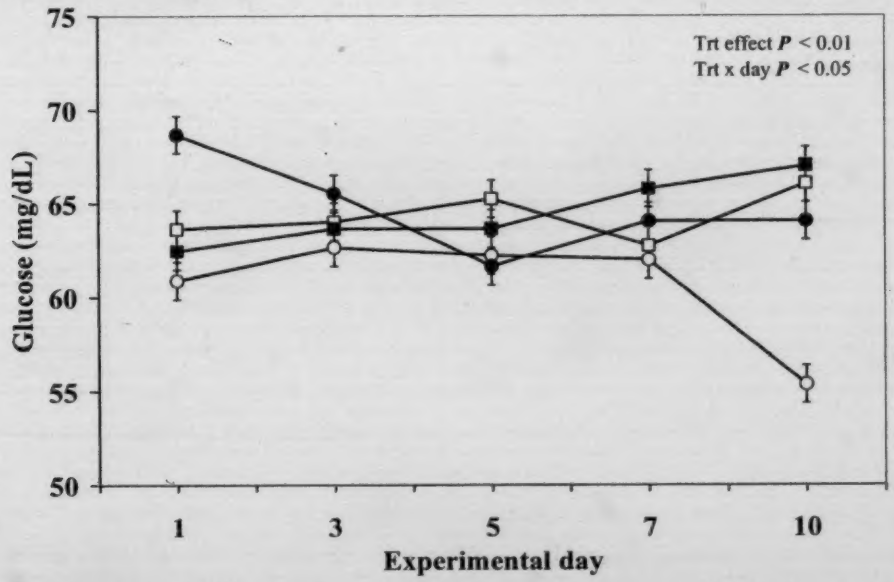

Figure 3.6 Concentration of glucose (mg/dL) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

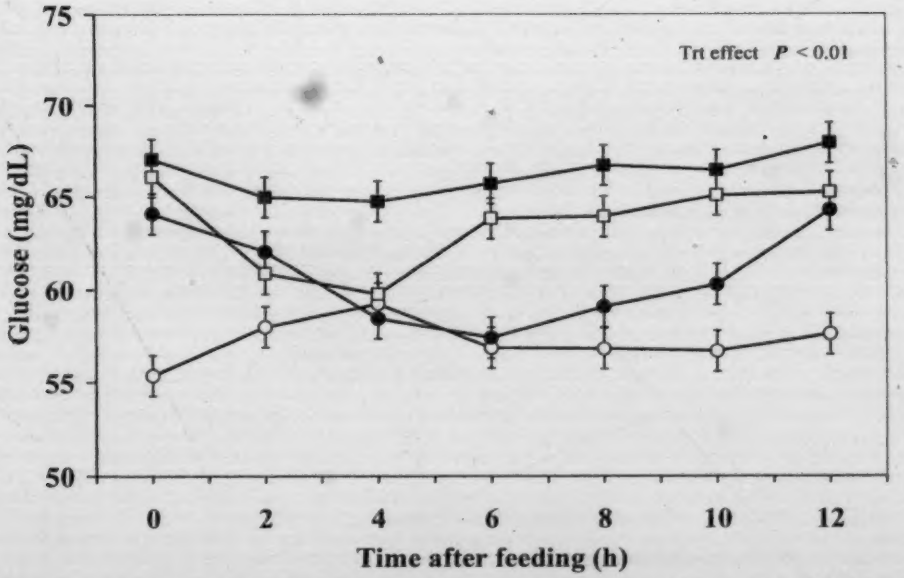

Figure 3.7 Concentration of glucose (mg/dL) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period at different time points after morning feeding.

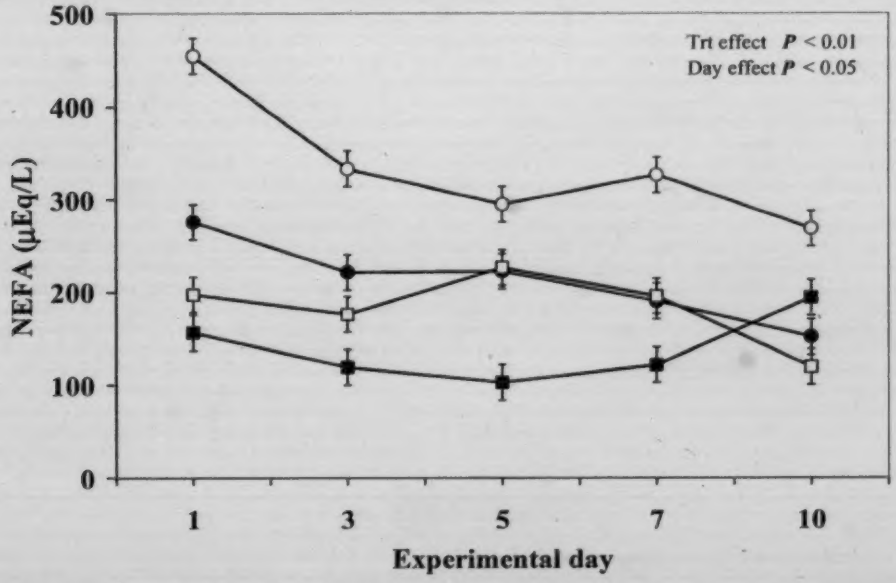

Figure 3.8 Concentration of NEFA (μEq/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

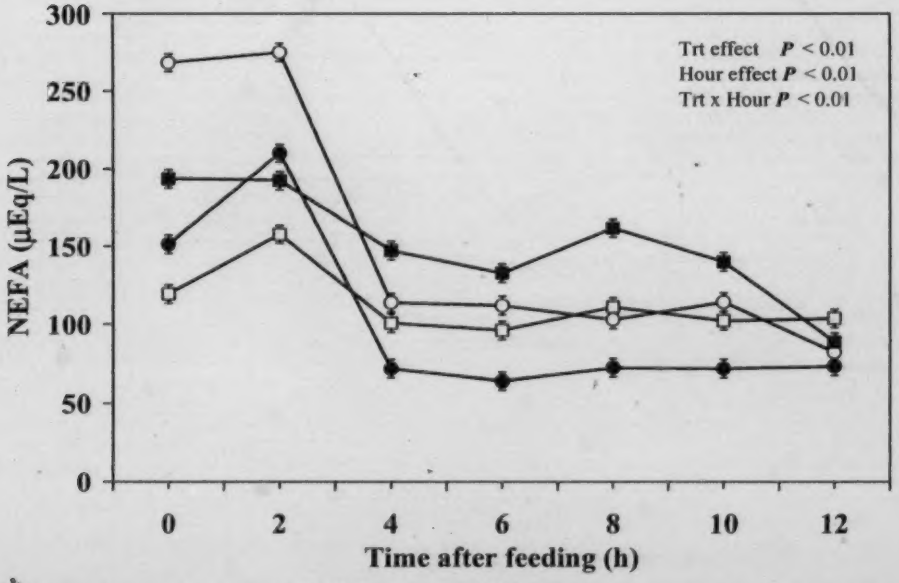

Figure 3.9 Concentration of NEFA (μEq/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period at different time points after morning feeding.

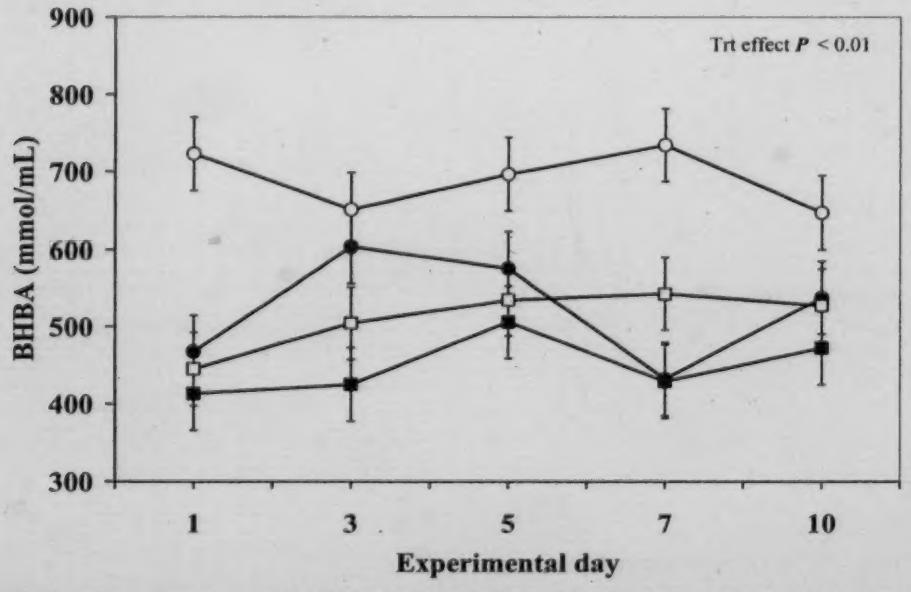

Figure 3.10 Concentration of BHBA (mmol/mL) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

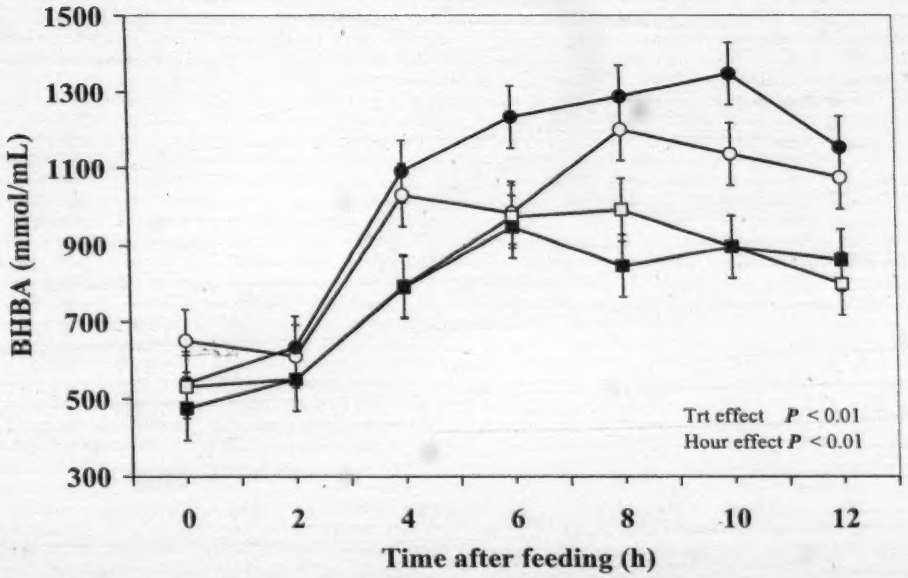

Figure 3.11 Concentration of BHBA (mmol/mL) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period at different time points after morning feeding.

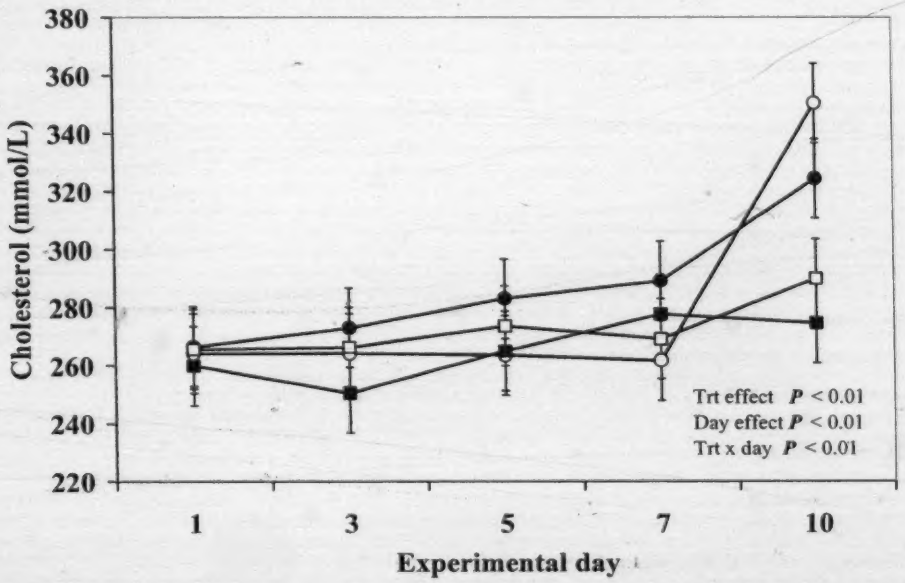

Figure 3.12 Concentration of cholesterol (mmol/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

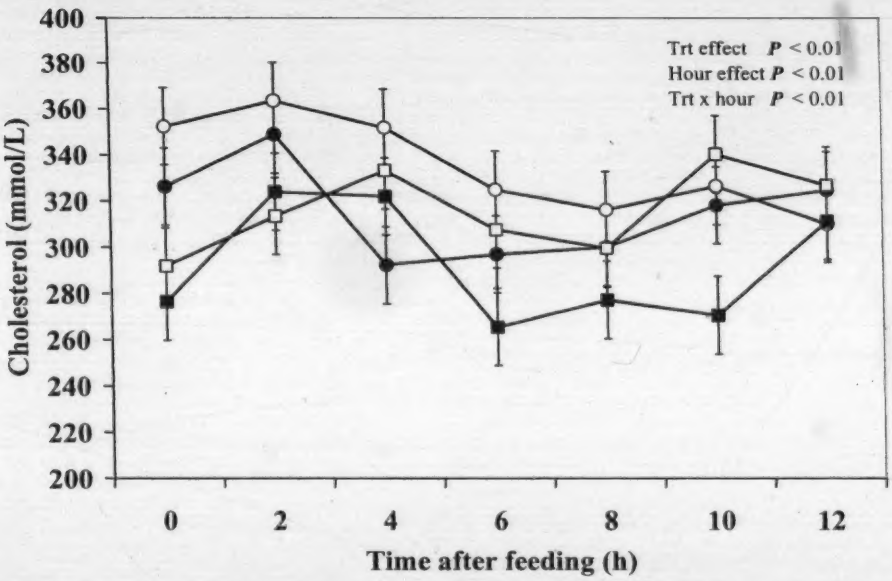

Figure 3.13 Concentration of cholesterol (mmol/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period at different time points after morning feeding.

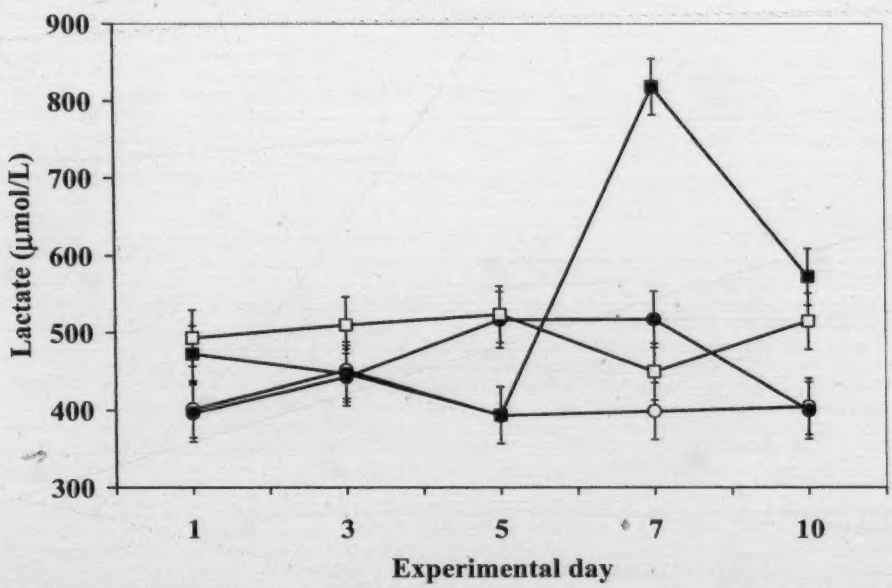

Figure 3.14 Concentration of lactate (µmol/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

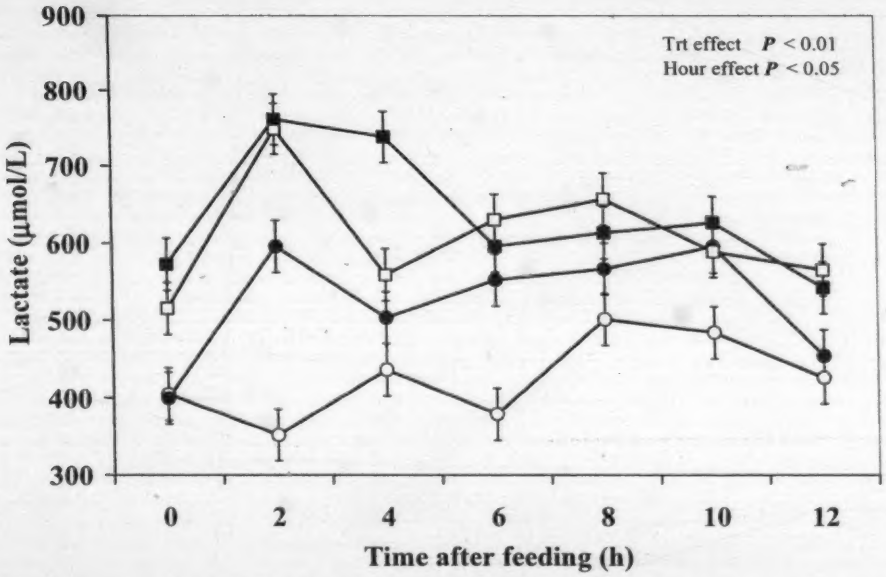

Figure 3.15 Concentration of lactate (µmol/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period at different time points after morning feeding.

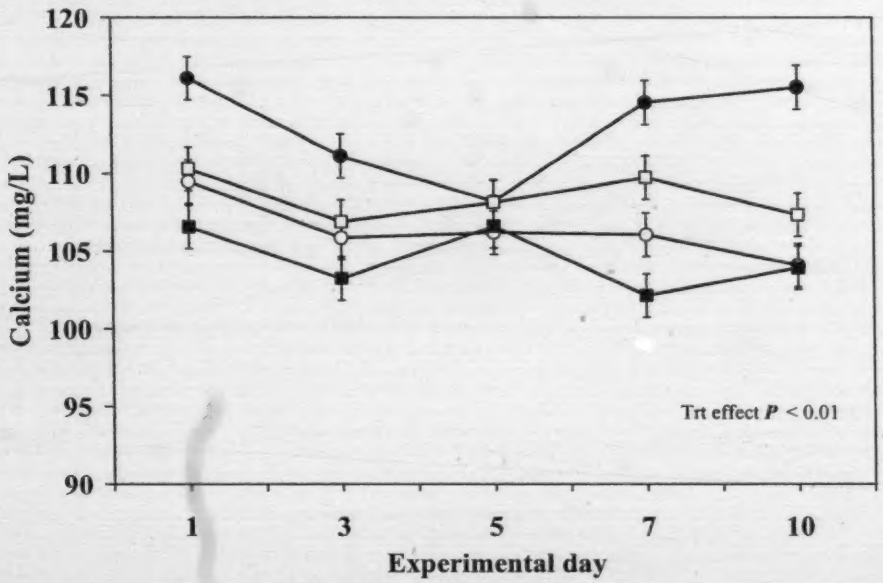

Figure 3.16 Concentration of calcium (mg/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

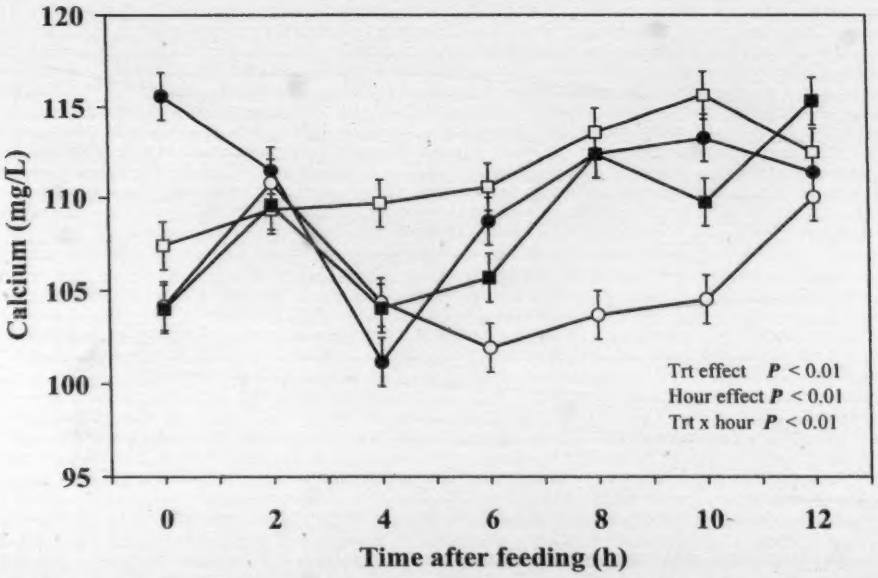

Figure 3.17 Concentration of calcium (mg/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period at different time points after morning feeding.

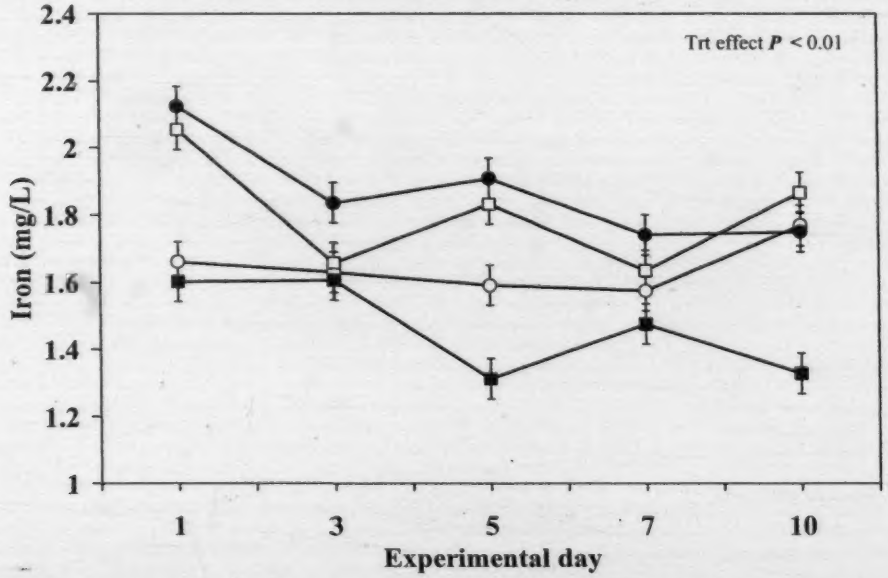

Figure 3.18 Concentration of iron (mg/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

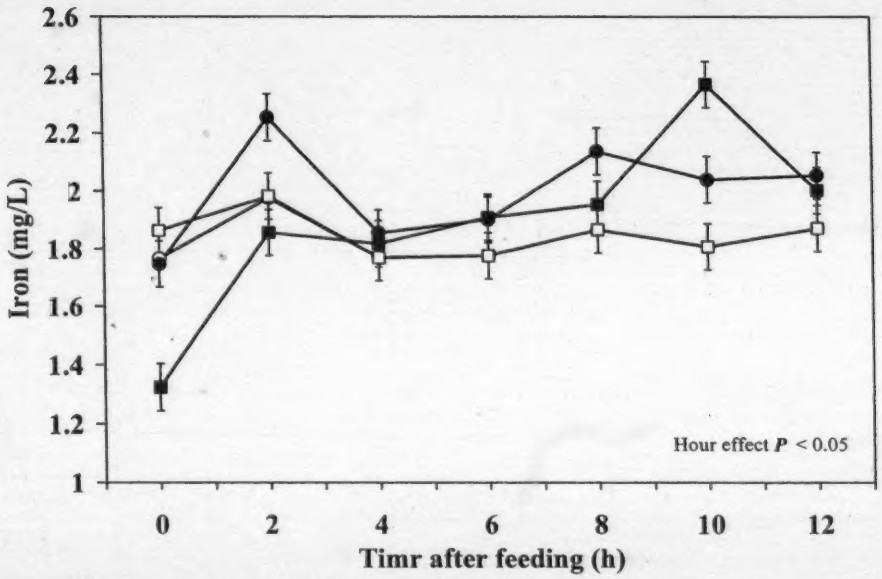

Figure 3.19 Concentration of iron (mg/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period at different time points after morning feeding.

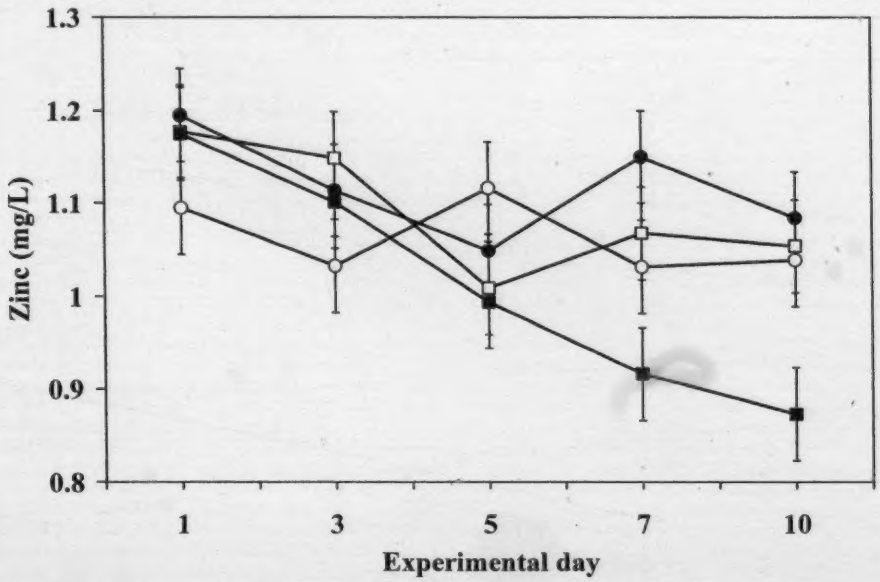

Figure 3.20 Concentration of zinc (mg/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

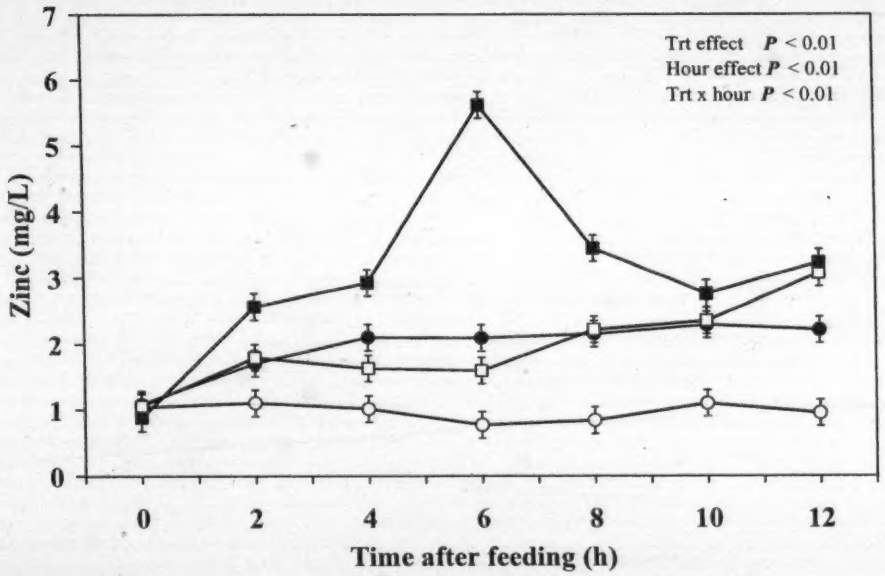

Figure 3.21 Concentration of zinc (mg/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period at different time points after morning feeding.

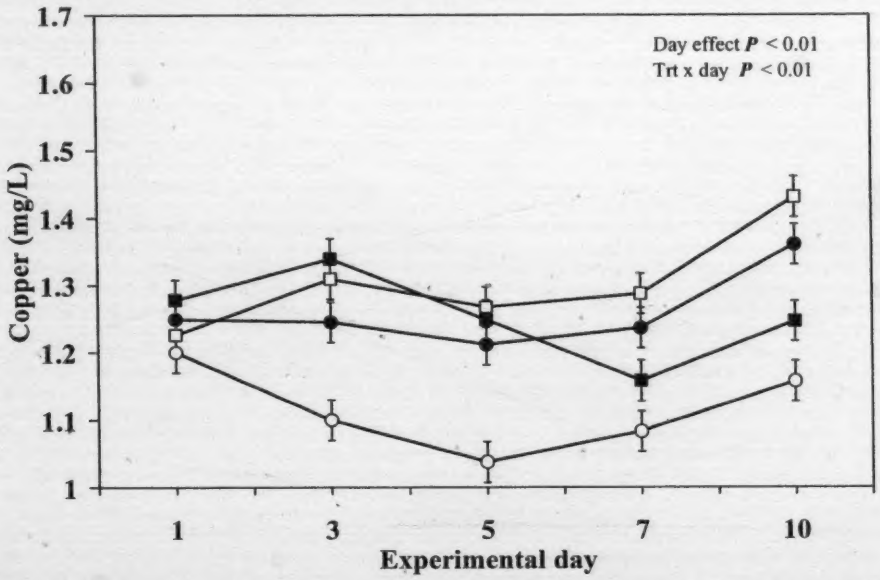

Figure 3.22 Concentration of copper (mg/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

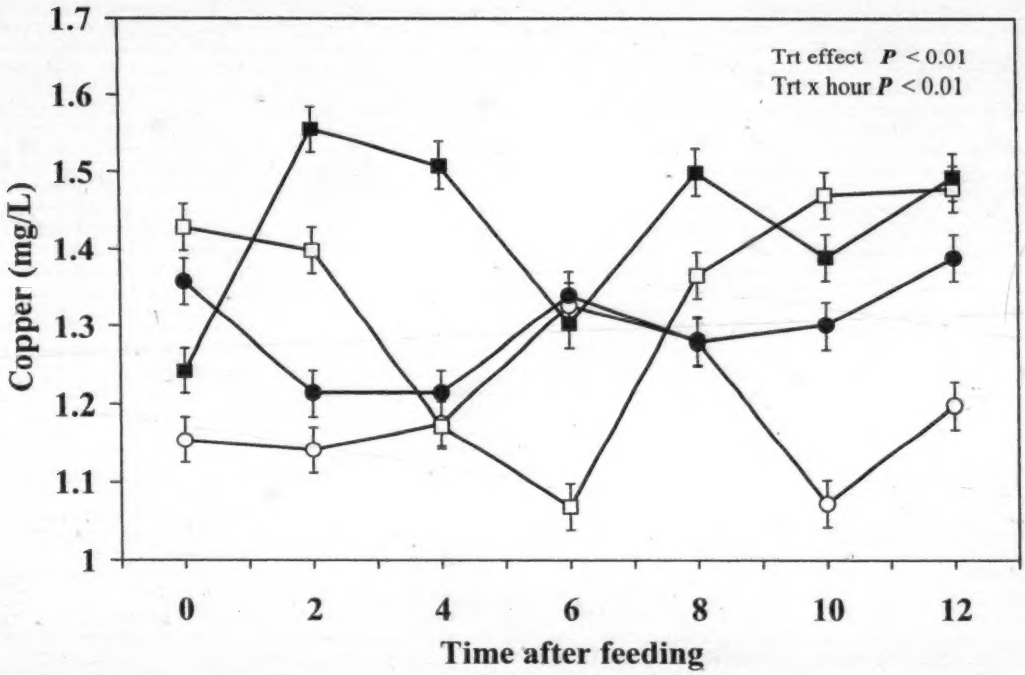

Figure 3.23 Concentration of copper (mg/L) in the plasma of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period at different time points after morning feeding.

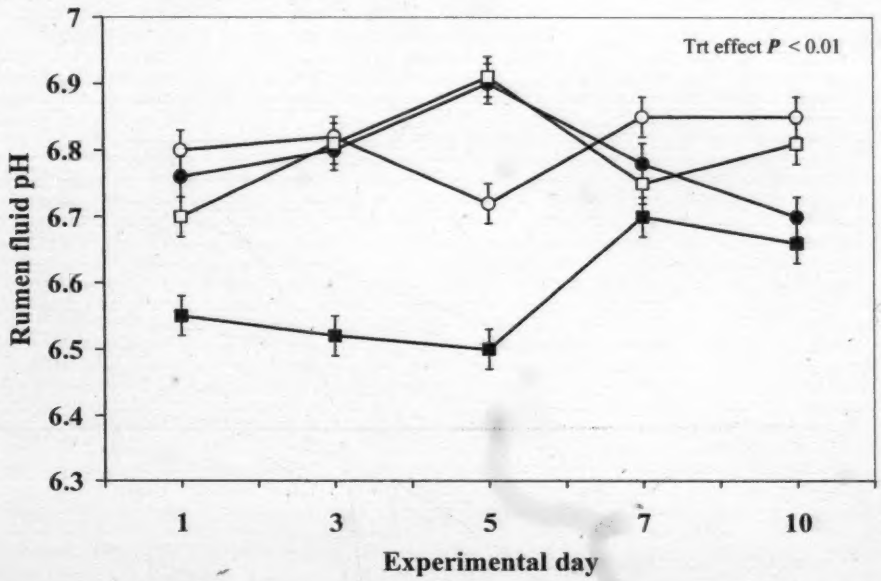

Figure 3.24 Rumen fluid pH of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

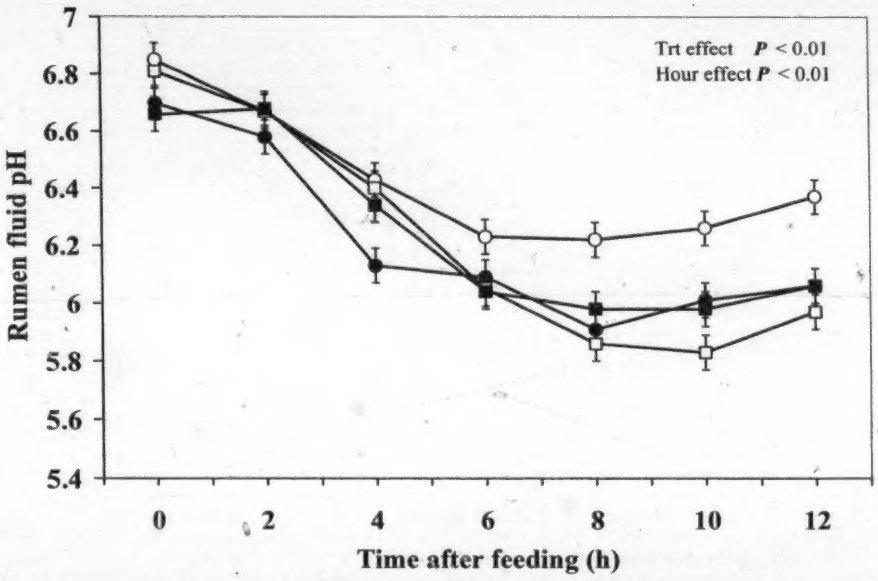

Figure 3.25 Rumen fluid pH of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period at different time points after morning feeding.

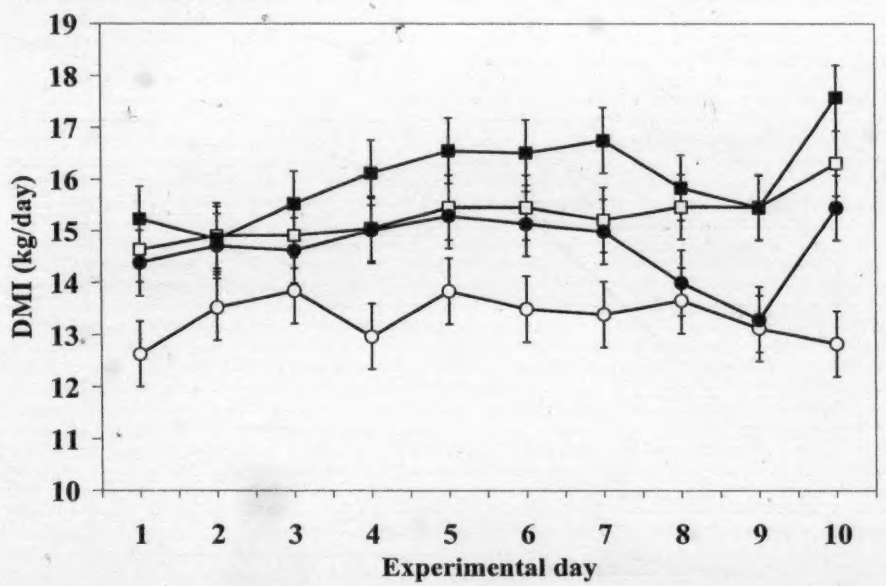

Figure 3.26 Dry matter intake of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

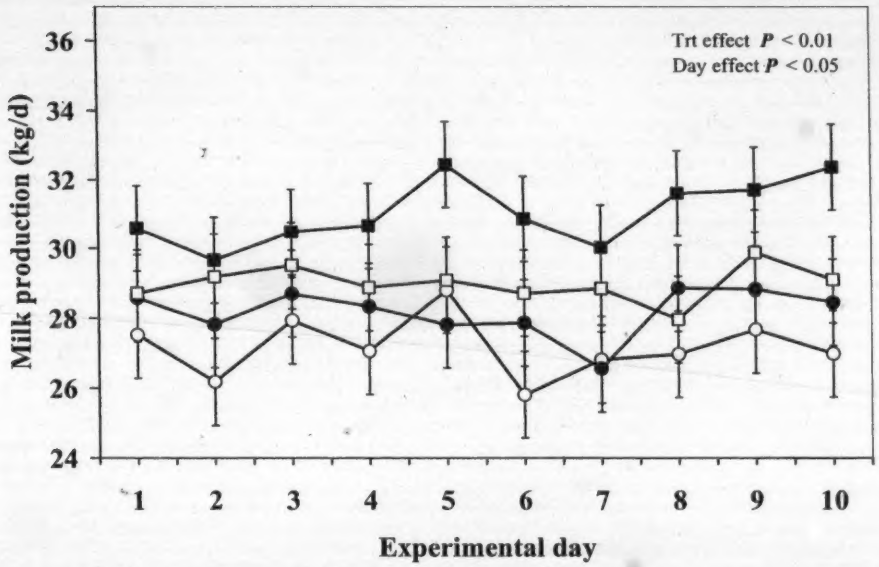

Figure 3.27 Milk production of early lactating (~ 60 DIM) cannulated Holstein dairy cows (n = 8) fed 0% (○), 15% (●), 30% (□), or 45% (■) barley grain in a 4 x 4 Latin square design with 11-d adaptation period and 10-d measurement period.

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Chapter 4

Effects of Acidic pH and Presence of Lipopolysaccharide (LPS) from *Escherichia coli* B:055 on Permeability of Rumen and Colon Tissues to Mannitol and LPS

4.1. Introduction

Feeding dairy cows diets high in starch (i.e., grain) is associated with alterations in the ruminal pH (i.e., acidosis) and the microbial ecology. Several researchers have induced acute or subacute acidosis by repeated doses of highly fermentable carbohydrates (Gentile, 1986; Harmon, 1985; Nagaraja and Town, 1990). The critical pH threshold of the rumen is < 5.0 during acute acidosis and < 5.5 during subclinical acidosis (Nagaraja, 1990). As highly fermentable carbohydrates are introduced to the diet, ruminal VFA production increases and pH starts to decrease. For example, Harmon et al. (1985) showed that intraruminal dosing with glucose decreased pH to < 4.5 within 14 h post-feeding.

The ruminal microbial profile also changes during feeding of high-grain diets. For example, *S. bovis* and lactic acid production both increase and there is a concomitant decrease in lactate utilizers such as *M. elsdenii* and *S. ruminantium*, and microorganisms that synthesize lactic acid outnumber those that utilize lactic acid (Russell, 1986; Strobel, 1986). As pH continues to drop, growth of *S. bovis* is also impeded; however, lactobacilli fill this niche and continue to produce lactic acid as pH drops.

Several studies have reported that acidosis is associated with a significant increase in the amount of endotoxin in the rumen (Dougherty and Cello 1949; Nagaraja et al., 1978; Anderson et al., 1994). There are also indications that endotoxin translocates into the bloodstream and causes a variety of metabolic and immunologic alterations to the host (Dougherty, 1975; Andersen et al., 1994). Moreover, endotoxin has been suggested to play a role in several metabolic disorders in cattle such as laminitis, abomasal displacement, fatty liver, and sudden death syndrome (Cappock 1972; Cappock 1974; Dougherty et al., 1976; Nagaraja 1979; Livesey and Fleming, 1984; Gerloff, 1986; Ametaj et al., 2005). However, the precise mechanism and the favorable conditions that helps translocation of endotoxin into the blood circulation is not known and attempts to measure endotoxin in the systemic circulation after introduction into the rumen has not been fruitful (Nagaraja, T. G.; personal communication).

Ruminal alterations also are associated with a variety of systemic changes such as decreased ruminal motility, stasis, rumenitis, and hyperkeratosis (Dirksen, 1989). Other events associated with acidosis include increased ruminal osmotic pressure, decreased extracellular volume resulting in dehydration, decreased cardiac output, decreased peripheral perfusion, decreased renal blood flow, shock, and death. In many dairy operations the challenge is not the acute acidosis, but rather subacute acidosis, whereby very little accumulation of lactic acid is detected in the rumen; however, the pH decreases. Daily episodes of pH < 5.5 for given periods ultimately predispose cattle to low grade subclinical acidosis. Symptoms include erratic appetite, body weight loss, diarrhea and lameness (Gentile, 1986; Norlund, 1995).

Although the clinical signs of acidosis are related to increased lactic acid concentration in the rumen it is not clear whether other compounds are involved in the symptomatology of ruminal acidosis. A potential candidate to be considered is endotoxin. Administration of LPS to ruminants is associated with similar clinical signs as observed during acidosis. Lipopolysaccharide, a structural component of the outer membrane of all Gram-negative bacteria, is a highly proinflammatory molecule and is shed from Gram-negative bacterial surface after bacteriolysis or during periods of rapid bacterial proliferation (Rietschel et al., 1994). Modern animal husbandry systems feed rations containing high quantities of grain for high milk and meat production. However previous studies have indicated that high-grain diets are associated with several fold increase in the concentration of endotoxin in the ruminal fluid and its translocation into the bloodstream (Anderson et al., 1994; Gozho et al., 2005; Gozho et al., 2006).

Although some of the clinical and biochemical symptoms exhibited in these diseases are similar to the classical signs of endotoxemia, decisive evidence of the presence of endotoxin in these situations is lacking. This arises the question as to whether endotoxin translocates into blood and causes effects or the toxicity is caused by an exaggerated host response to endotoxin (Morrison and Ulevitch, 1978; Green and Adams, 1992).

The gastrointestinal barrier comprised of the mucus secreted by the goblet cells and the epithelial cells lining the gastrointestinal tract plays a prominent role in protecting the body. An essential element in this process is the formation of tight junctions between adjacent epithelial cells making up the epithelial sheet. In some organs, notably the gut

and the lung, this barrier function is also important to prevent systemic contamination by microbes and toxins that are present in the external environment (Stevenson, 1999). Impairment of gastrointestinal barrier function during sepsis leads to massive translocation of bacteria and their lysis products such as endotoxin (O'Boyle et al., 1998; Naaber et al., 2000).

The gastrointestinal tract of ruminants is an important source of endotoxin as indicated by reports from Anderson et al. (1994) and Nagaraja et al. (1978). Normally, the barrier function of the intestine prevents the entry of endotoxin into the systemic circulation, but endotoxin can activate the resident mucosal leukocytes in the gut directly or indirectly through the induction of cytokines. Primed leukocytes leave the gut and on activation in distant organs may cause damage. This priming or activation of leukocytes in the gut mucosa can also cause an inflammatory reaction and may lead to systemic consequences (Moore et al., 1994). Loss of gut barrier function or increased permeability of the gastrointestinal mucosa has been implicated in the development of systemic inflammation and distant organ failure in humans and laboratory animals (Deitch, 1992). Considerable body of evidence suggests endotoxin or proinflammatory cytokines to be involved in the loss of mucosal barrier function (Salzman, 1995; Szabo, 1995). Endotoxin-induced gut injury and the consequent increased permeability or loss of barrier function leads to translocation of bacteria and bacterial products resulting in the development of sepsis (Deitch, 1992). Endotoxin-induced loss of gut barrier functions is thought to occur through different mechanisms. There is considerable amount of evidence to support that endotoxin-induced loss of gut barrier functions results from ischemia-reperfusion injury

to the gut (Fink et al., 1989; Navaratnam et al., 1990). Exaggerated NO production induced by endotoxin or proinflammatory cytokines has been related to the loss of gut barrier function (Weer et al., 1997; Rixen et al., 1997). Nitric oxide, at normal physiological conditions, has beneficial effects in the gut like maintaining mucosal perfusion, inhibiting neutrophil adherence to the endothelium via the down-regulation of surface expression of the leukocyte adhesion molecule CD11/CD18, blocking of platelet adhesion, abrogating the release of inflammatory mediators such as platelet activating factor and histamine by stabilizing intestinal mast cells (Beckman et al., 1990; Kubes et al., 1991). The enzyme nitric oxide synthase that regulates the production of NO exist in two forms, the constitutive and inducible isoforms. The constitutive NO synthase produces small amounts of NO and is actually protective by preventing mast cell activation, platelet adhesion, and acting as antioxidant (Caplan et al., 1994; Kanner et al., 1991; Kanwar et al., 1994). The second isoform which is the inducible NO synthase is induced by both endotoxin and proinflammatory cytokines (Caplan et al., 1994). Prolonged exposure of cells to high amounts of NO cause cellular damage in paracrine or autocrine fashion (Caplan et al., 1994). The regional blood flow is also affected eventually leading to loss of gut barrier function and increased gut permeability (Doughty et al., 1998; Unno et al., 1997).

Acidotic conditions also have a profound effect on the barrier function by altering the permeability of the gut epithelial cells. All acidic milieu involving mucosal, intracellular, and acidic blood perfusing the tissue results in a deranged permeability of gut epithelial cells (Salzman et al., 1994; Unno et al., 1997). Studies conducted in pig reveal that

mucosal acidosis of pig ileal epithelium results in increased permeability and that the hyperpermeability occurs even in absence of tissue hypoperfusion or cellular hypoxia (Salzman et al., 1994). Similarly, Nylander et al. (1989) instilled concentrated hydrochloric acid into feline small intestine and observed that the extreme acidic conditions greatly increase the epithelial permeability. Even though such acidic conditions are unlikely to occur in small intestines these results give an insight of the effects of acidosis on permeability of epithelial cells in the gastrointestinal cells. In vitro studies using cultures of intestinal Caco-2_{BBe} epithelial monolayers also show an increased permeability to hydrophilic molecules like mannitol, fluorescein sulfonic acid, and fluorescinisothiocyanate-labelled dextran under acidic conditions (Menconi et al., 1997). The hyperpermeability of acidosis results mainly from injury to the integrity of cell membrane, which is disrupted under acidotic conditions (Menconi et al., 1997).

Acidosis-induced hyperpermeability is also dependent on the duration of exposure to low-pH environment. Studies with intestinal Caco-$_{2BBe}$ epithelial monolayers indicate that increased permeability occurs after 2h of incubation at a pH of 5.43 and the derangement in permeability was consistently larger after 24 h of incubation (Menconi et al., 1997). Ultrastructural studies of cells exposed to low pH indicate dilated tight junctions and decreased amount of electron dense material due to the loss of tight junction associated protein ZO-1 (Menconi et al., 1997). Acidosis-induced hyperpermeabilty is reversible and the lost function regains when shifted from the acidic medium. The restoration of normal permeability involves cellular proliferation to replace damaged cells and repair of the preexisting cells (Menconi et al., 1997). Acidic conditions also potentiate the effects of

NO on intestinal permeability. Nitric oxide is capable of increasing the permeability of Caco-2_{BBe} monolayers when the extracellular pH of the culture medium is maintained at 7.4. But the effect is more pronounced under mildly acidic conditions of pH 6.5-7 (Unno et al., 1997).

Since the acidic rumen environment and presence of endotoxin render the ruminal epithelium susceptible to injury (Brent, 1976; Enemark et al., 2002; Kleen et al., 2003), we hypothesized that exposing rumen and colon tissues of cattle to acidotic pH and LPS will alter their permeability and lead to translocation of LPS through these tissues. To test our hypothesis we conducted an *in vitro* experiment in Ussing chamber. The experiment was performed at the Division of Gastroenterology, University of Alberta.

4.2. Materials and methods

4.2.1. Sample collection and preparation. Rumen and colon samples for the experiment were collected from an Edmonton-based abattoir (Edmonton Custom Packers). Within 10 min after slaughter of feedlot steers, a 10 cm^2 area of rumen tissue and 15 cm length of colon were excised from the gastrointestinal tract, washed with cold Ringer's lactate solution and stored in an amino acid solution until processing and mounting the tissues in the Ussing chamber. Composition of the amino acid solution and Ringer's lactate are given table 4.1 and 4.2, respectively. The amino acid solution is used to preserve human torgans prior to transplantation and was prepared and provided by Dr. Churchill's laboratory at the Department of Surgery, University of Alberta. Samples were carried to the laboratory in the cold amino acid solution (4-5^{0}C) in a cooler and during

transportation the samples were aerated with a syringe to facilitate respiration of the tissues. On reaching the lab, samples were prepared for mounting in an Ussing chamber on a glass surface which was kept cold by ice. The serosal and muscular layer was peeled off carefully from both rumen and colon samples and the maximum possible fibrous tissue was removed from the mucosa without injuring the tissue. Tissues were cut into squares (approximately 2 cm^2) for mounting in the Ussing Chamber to have an exposed area of 1.76 cm^2. For each experiment, samples from 8 animals were used. The time from collection till mounting of tissues in the chambers was approximately 40 min.

4.2.2. Ussing chamber experiment After turning on the water heater and assembling the lucite chambers between the supporting screws, 10 mL of Ringer's lactate was added to both reservoirs of the Ussing chamber. The quantity of each reagent for preparation of Ringer's lactate is shown in table 4.2. Any air bubble in the lucite chamber was removed by adjusting the leads. To both the reservoirs of Ussing chamber 200 µL of glucose solution was added and chambers were connected to a 95% O_2/5% CO_2 airlift. Once the Ringer's lactate in the system attained a temperature of 39^0 C, the resistance button was adjusted to bring the fluid resistance to zero. The electricals were then switched to standby and the tubular ends of the chamber to the lucite chamber was clamped on both the serosal and the mucosal reservoirs and the right half of the lucite chamber was removed. The prepared colon or rumen tissue was mounted on the pins of the lucite chamber with the mucosal side in contact with the lucite chamber and the chambers were then reassembled. Once the tissues were mounted on all the six Ussing chambers the Ringer's lactate in the mucosal side of the chambers was drained out and replaced with

Ringer's lactate whose pH was adjusted to 4.5, 5.5, or 6.5 for rumen samples and 5.5, 6.5, and 7.4 for colon samples. After replacing the pH adjusted Ringer's lactate in the mucosal side, 10 µL of ^{3}H-mannitol (525.4 GBq/mmol) was added to the mucosal side to serve as a permeability marker. Also, 500 µg/mL of LPS from *E. coli* B:055 was added to the solution on the mucosal side of the chambers to evaluate the effects of LPS in altering the permeability and also to see if LPS translocates from the mucosal to the serosal side. The system was allowed to equilibrate for 15 min and at 15 min two fluxes (hot) (100 µL each) was taken from the mucosal side of the chambers to detect the initial concentration of ^{3}H-mannitol. At time 20, 25, 30, and 35 min 1 mL and 200 µL of fluxes (cold) were taken separately from the serosal compartment for detection of ^{3}H-labeled mannitol and LPS. At the end of 40 min, another two fluxes (100 µL each) were taken from the mucosal side of the chambers to detect the available concentration of ^{3}H-mannitol in the mucosal side after translocation into the serosal side. After each sampling, electrical measurements were made to determine the isoelectric current and the potential difference.

4.2.3. Laboratory analyses

Determination of ^{3}H-mannitol

Four mL of scintillation fluid was added to the vials for detection of ^{3}H-mannitol, since ^{3}H is a low energy beta emitter which cannot be detected without adding a scintillation fluid. The scintillation fluid emits a fluorescent pulse on absorbing radiation and the amount of radiation was measured as counts per minute (cpm) in a liquid scintillation counter (Beckman Instruments., Irvine, CA). The quantity of translocated ^{3}H-mannitol was detected from the cpm of hot and cold fluxes using an excel program.

Determination of LPS

Lipopolysaccharide from *E. coli* B:055 in the serosal side of the chamber was quantified indirectly by determining the amounts of C_{12}-lauric and C_{14}-myristic fatty acids in the solution (Silipo et al., 2002). The fatty acid composition of LPS from *E. coli* is described by Datta et al. (1999). For detection of LPS 100 µL of the sample was freeze-dried. The freeze-dried samples were methylated by adding 1 ml of methanolic HCL 3N (Supelco., Bellefonte, PA). The samples were then vortexed before placing in a water bath at 50^0C and shaken every 5 min for 30 min. Methylated fatty acids were extracted by adding 50 µL of H_2O and 3 mL of hexane and shaking vigorously for 20 sec. The top hexane portion was then removed using a disposable Pasteur pipette (Fisher Scientific., Fair Lawn, NJ) and dried under liquid nitrogen. The dried fatty acids were again dissolved in 150 mL of hexane containing internal standard (2 mg C_{17} in 1000 mL of hexane) and injected into 50 mm x 0.25 mm film thickness capillary column (Supelco., Bellefonte, PA) in a Varian 3400 Gas Chromatography equipped with Varian 8100 auto sampler. Helium was used as the carrier gas at a rate of 1.5 mL/min. Injector temperature was programmed from 50^0C to 230^0C at 150^0C/min with a run time of 36 min. Detector temperature was set at 230^0C and peak area integration for fatty acids were made using Galaxy software (Varian Inc., Walnutcreek, CA).

4.2.4. Statistical analyses

To determine the effects of presence of LPS and acidic pH on permeability to ^{3}H-mannitol and translocation of LPS through rumen and colon tissues, data were subjected to statistical analysis using the SAS (1989) General Linear Model procedure using the following model:

$Y_{ijk} = \mu + T_i + P_j + TP_{ij} + e_{ijk}$

where μ is the population mean, T_i is the fixed effect of treatment i, P_j is the effect of pH j. TP_{ij} is the effect of treatment by time interaction and e_{ijk} is the residual error. The PDIFF option was used in each of the comparisons. Significance was declared at $P < 0.05$.

4.5. Results

4.5.1. Permeability of rumen and colon tissues to ^{3}H-mannitol

Acidic pH and presence of LPS increased permeability of rumen tissue to ^{3}H-mannitol ($P < 0.05$) (Fig 4.1). In general, presence of LPS increased permeability of rumen tissue to ^{3}H-mannitol with a permeability of 11.7 nm/cm^2/h compared to 4.9 nm/cm^2/h in the controls. An interaction between LPS and pH was also obtained with respect to permeability of rumen tissue to ^{3}H-mannitol ($P < 0.01$). Permeability increased by 6-fold at a pH of 4.5 in the presence of LPS (4.10 vs. 25.09 nm/cm^2/h). However, presence of LPS did not increase affect permeability of rumen tissue to ^{3}H-mannitol at pH values of 5.5 and 6.5.

Neither acidic pH nor presence of LPS affected permeability of colon tissues to ^{3}H-mannitol (Fig 4.2). Although we observed higher numerical permeability to ^{3}H-mannitol

at pH 5.5 (83.05 nm/cm^2/h) compared to pH 6.5 (31.73 nm/cm^2/h) and pH 7.4 (26.5 nm/cm^2/h), the difference did not reach statistical significance. Also, no interaction between pH and LPS was evidenced, although a numerical increase in permeability to ^{3}H-mannitol was observed in the presence of LPS (63.1 nm/cm^2) compared to controls (31.07nm/cm^2). At pH 5.5, the permeability of the colon to ^{3}H-mannitol in the control and the LPS treated groups tended to differ (137.01 vs 29.1 nm/cm^2/h); however, the difference did not reach significant level.

4.5.2. Permeability of rumen and colon tissues to LPS

There was translocation of LPS from the mucosal to the serosal side of the Ussing chamber in all groups treated with LPS ($P < 0.01$). However, exposure of rumen tissues to different acidic pH did not affect translocation of LPS and there was no difference in the quantity of LPS translocated between the different pH groups (Fig. 4.3). Also, no interaction between pH and LPS was obtained. Evidently, LPS was translocated from the mucosal to the serosal side of the Ussing chamber at all three pH values when the tissues were exposed to LPS ($P < 0.01$). All three pH values did not effect translocation of LPS through the colon tissues and no interaction between pH and LPS was obtained (Fig. 4.4).

4.6. Discussion

Subacute ruminal acidosis and ARA are common pathologies in dairy cows fed rapidly fermentable carbohydrates. Cows affected by SARA or ARA have erratic eating, low rumination rate, lower milk production, and milk fat content, mild diarrhea and foamy feces as well as appearance of undigested grain in the feces. If not treated, affected cows die of rumen stasis and other complications like laminitis or abomasal disorders. The

reason for the general aggravation of cow's health during ruminal acidosis is not very well understood. Although lactic acid and translocation of toxic compounds like endotoxin have been blamed for the general clinical pathology there is no evidence about conditions that favor absorption of toxic compounds into the host's blood.

Generally, physiological rumen and colon pH values range between 6.5 and 7.4, respectively (Diez-Gonzalez et al., 1998; Garrett, 1999; Russell, 2000). During SARA these variables decline to 5.5 and 6.5 for rumen and colon tissues, respectively (Diez-Gonzalez et al., 1998; Garrett, 1999; Russell, 2000). Furthermore, during ARA these values decrease even more to 4.5 and 5.5, respectively (Diez-Gonzalez et al., 1998; Garrett, 1999; Russell, 2000). Feeding cattle high-grain diets also is associated with increased concentrations of endotoxin in the rumen. Nagaraja et al. (1978) report rumen fluid endotoxin concentrations ranging between 400 and 900 μg/mL in feedlot cattle fed a diet containing 90% grain. Commonly, early lactating dairy cows are fed barley grain at proportions that reach 45% of the diet (DM-basis). In our Ussing chamber experiment, we used an average endotoxin concentration of 500 μg/mL. The present study was designed to investigate whether pH values similar to those observed during SARA and ARA and concentrations of LPS similar to those observed during feeding of high-grain diets would affect permeability of rumen and colon tissues to ^{3}H-mannitol and LPS.

The main finding of this study was that in the presence of LPS and at acidic pH values (i.e., 4.5) there was an increased permeability of rumen tissue to ^{3}H-mannitol. On the contrary, in presence of LPS and at normal pH values (i.e., 6.5) and pH values similar to SARA (i.e., 5.5) rumen permeability to ^{3}H-mannitol was not affected. Another significant

finding of this research was that permeability of colon tissues to ^{3}H-mannitol was several fold higher in colon tissues immersed in Ussing chamber solutions at pH values of 5.5 and presence of LPS; however, the difference did not reach significance level. While LPS and acidic pH act separately to increase the permeability of the mucosa, the increased permeability observed at acidic pH and presence of LPS suggests that the two factors combine to further enhance the permeability of mucosa to ^{3}H-mannitol. In the present study, we used ^{3}H-mannitol to study changes in permeability. ^{3}H-mannitol, with a molecular weight of 182 daltons and crosssectional diameter of 0.67 nm, crosses minimally epithelial layers and is commonly used as an intestinal permeability probe (Hollander et al., 1988).

The mechanism by which low pH and presence of endotoxin enhanced permeability of rumen and colon tissues to ^{3}H-mannitol is not known; however, several mechanisms might be involved. Studies examining the effects of acidic pH on mucosal permeability reveal that acidic environment affects the permeability of mucosal epithelial layers by increasing production NO in enterocytes (Salzman et al., 19; Unno et al., 1997). Also, LPS has been shown to induce production of NO in various enterocytic cell lines in vitro (Dignass et al., 1995; Unno et al., 1995). Presence of high concentrations of NO may inhibit production of ATP and damage enterocytes (Unno et al., 1997). Our data indicated that, with rumen tissue, lowering the pH was able to increase the permeability to ^{3}H-mannitol. The increased permeability at a pH of 4.5 for rumen and of 5.5 for colon tissues in presence of LPS may be due to combined disrupting effects of LPS and acidic pH on the epithelial barrier functions.

Another significant finding of the present study was that translocation of LPS across the rumen and colon mucosa of cattle was not pH dependant. Lipopolysacharide from *E. coli* B:055 translocated through these tissues if present at the mucosal side. Our results confirm previous studies indicating that translocation of endotoxin through mucosal layers are not dependent on pH (Drewe et al., 2001).

Recently, Drewe et al. (2001) demonstrated that translocation of LPS can take place both by simple diffusion through transcellular pathway as well as by paracellular pathway and that translocation was not related to presence of Na^+ ions. The latter indicates that no active translocation of LPS occured. By disrupting the microtubule system, the above study indicated that translocation of LPS across the mucosa occurs by an unknown active endocytic pathway in addition to the paracellular pathway.

Another potential mechanism by which LPS translocates through epithelial layers is by down-regulation of structural proteins of tight junctions. The tight junctions are responsible for the barrier properties of the epithelia (Madara and Trier, 1982). Tight junctions are composed of integral membrane proteins, including occludins and claudins (Colegio et al., 2002). Exposure of epithelial cells to LPS has been shown to disrupt tight junctions by down-regulation of occludins (Yi et al., 2000). Thus, presence of LPS on the mucosal side of rumen and colon tissues, in our experiment, might have disrupted the epithelial tight junctions and contributed to increased permeability of rumen and colon tissues to ^{3}H-mannitol through paracellular pathway.

Our finding that LPS is translocated if present in the gut is reinforced by several other studies. For example, enteral administration of endotoxin in mice resulted in elevation of concentrations of LPS in blood compared to control mice (Schwarzenberg and Bundy 1994). *In vitro* studies with isolated gut segments, found that the flux of LPS was not proportional to concentration of LPS present in the mucosal side, but rather the transport system attained a saturated state with a threshold concentration (Nolan et al., 1977). In conclusion, it is possible that the concentration of LPS which we used in the present study was sufficient to activate the transport system and resulted in the translocation of LPS to the serosal side.

4.7. Conclusions

In conclusion, the permeability of rumen and colon tissues to ^{3}H-mannitol increased 4- to 5-fold in presence of LPS from *E. coli* B:055 and acidic pH values (pH 4.5 and 5.5 respectively). No translocation of ^{3}H-mannitol occurred when the pH on the mucosal side was 5.5 and 6.5 for rumen and 6.5 and 7.4 for colon. Translocation of LPS occurred across the rumen and colon tissues independendantly of pH. Further research is warranted to understand the mechanism(s) by which acidic pH and presence of LPS make the rumen and colon tissues leaky.

Table 4.1 Reagents and final concentration in the amino acid solution.

Reagent	Final concentration (mmol)	Quantity (g)/L
Lactobionate	20	7.1660
Glutamate	20	2.9420
Aspartic acid	20	2.6620
Arginine	10	1.7420
Proline	5	0.5755
Glycine	10	0.7507
Cysteine	5	0.6060
Asparagine	10	1.5010
Threonine	10	1.1910
Lysine	10	1.8260
Methionine	5	0.7460
Serine	10	1.0510
Leucine	5	0.6560
Isoleucine	5	0.6560
Valine	10	1.1710
Histidine	5	0.7760
Tyrosine	1	0.1812
Tryptophan	1	0.2042
Ornithine	5	0.8430
Trolox	1	1.0000
3-aminobenzamide	1	1.0000
HO butyrate	3	0.3783
Glucose	20	3.6040
N,N-Bis(2-hydroxyethyl)taurine	15	3.1980
Glutamine	35	5.1130
Adenosine	5	1.3360
Allopurinol	1	0.1361
HES buffer[1]	5%	50.0000

[1]Contained 20 mM HEPES–1 mM EDTA–250 mM sucrose

The pH of the solution was adjusted to 7.40 +/- 0.01 using 2M NaOH.

Table 4.2 Reagents and final concentration in Ringer's lactate solution.

Compound	Final concentration (mmol)	Quantity (g)/2L
$MgCl_2.6H_2O$	114.00	0.447
$CaCl_2.2H_2O$	5.00	0.367
NaCl	1.65	13.320
KCl	0.30	0.750
$NaHCO_3$	1.10	4.200
Na_2HPO_4	1.25	0.468
$NaH_2PO4.2H_2O$	25.00	0.094
Mannitol	1.00	0.364

The above reagents were added in the order listed and brought to final volume with double distilled H_2O and pH 7.4 using HCl or NaOH.

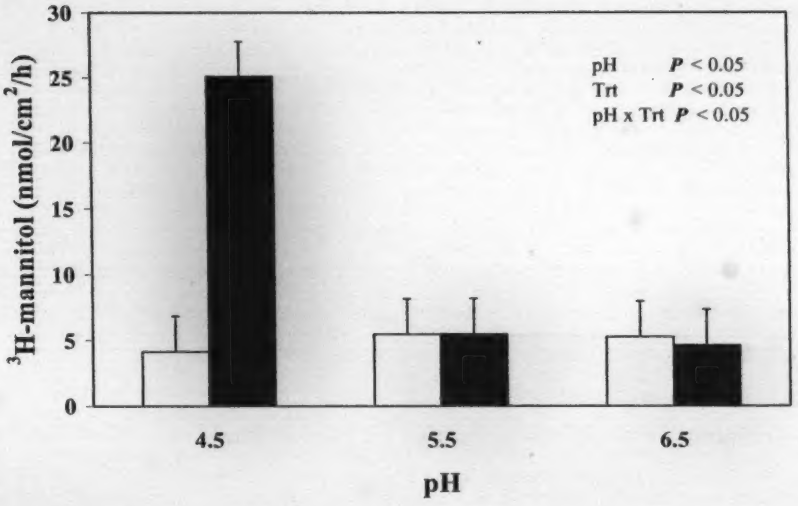

Figure 4.1 Permeability of rumen tissue (obtained from killed feedlot steers) to ^{3}H-mannitol under different pH and in presence (■) or absence (□) of lipopolysaccharide from *E. coli* B:055 in an Ussing chamber.

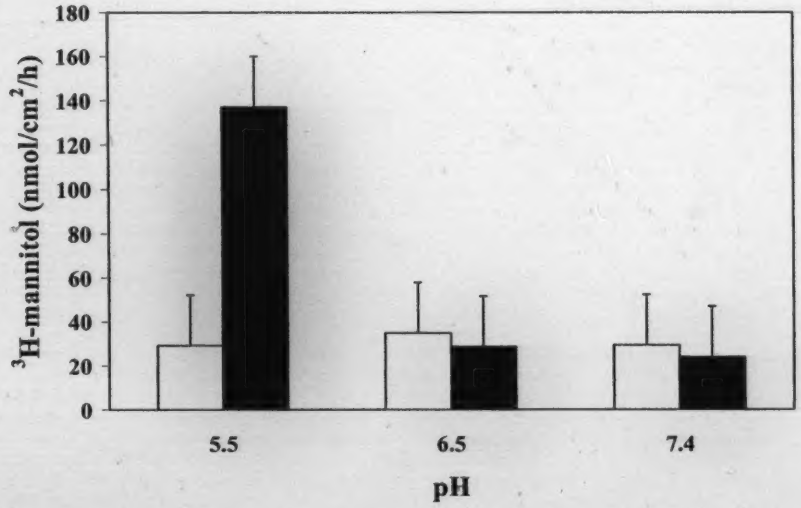

Figure 4.2 Permeability of colon tissue (obtained from killed feedlot steers) to ^{3}H-mannitol under different pH and in presence (■) or absence (□) of lipopolysaccharide from *E. coli* B:055 in an Ussing chamber.

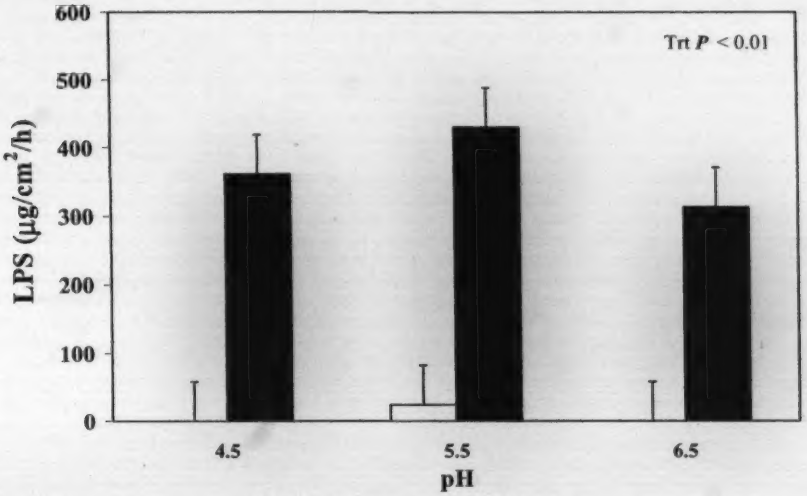

Figure 4.3 Translocation of lipopolysaccharide (LPS) from *E. coli* B:055 across rumen tissue (obtained from killed feedlot steers) under different pH and when present (■) or not present (□) in the mucosal reservoir of Ussing chamber.

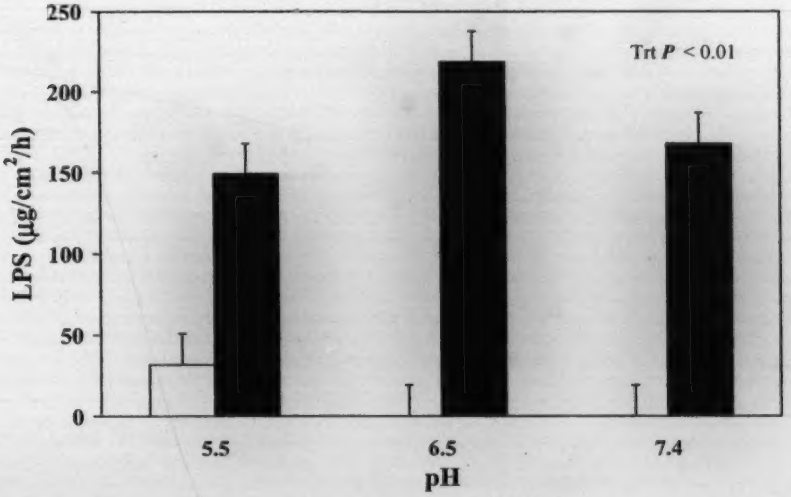

Figure 4.4 Translocation of lipopolysaccharide (LPS) from *E. coli* B:055 across colon tissue (obtained from killed feedlot steers) under different pH and when present (■) or not present (□) in the mucosal reservoir of Ussing chamber.

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